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(54) Title: ORGANISM AND METHOD FOR METAL RECOVERY, REMEDIATION AND SEPARATION

(57) **Abstract**

In the present invention, the capacity of the green alga, *C. reinhardtii*, to produce the metal-binding factors, in response to stress induced by the heavy metal Cd (Howe *et al.* 1992) has been addressed. Gekeler and coworkers (19B8) reported that *C. reinhardtii* accumulates phytochelatins, with n values range from 2 to 4, in response to Cd-induced stress. Howe *et al.* (1992) extended this observation by showing that newly synthesized Cd-induced peptides assemble into Cd-binding peptide complexes that chelate up to 70 % of the intracellular Cd ions in extracts of Cd-treated cells.

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Organism and Method for Metal Recovery, Remediation and Separation

This application claims the benefit of U.S. Provisional Application No. 60/087,374, filed May 28, 1998, incorporated herein by reference.

Technical Field

The present invention is in the field of metal recovery, remediation and separation.

Background

Surface soils and water are contaminated at many industrial and mining locations throughout the world with radionuclides, heavy metals, organic pollutants, as well as mixtures of these contaminants. Heavy metals (e.g. cadmium, cobalt, copper, lead, zinc) pose particular problems for environmental remediation because of their long term stability; that is, they must be sequestered from the media they have contaminated. Even though contamination levels are often relatively low, the limits of contamination allowed by the US Environmental Protection Agency (EPA) are usually less than 1.0 ppm (Aksu, Z. *et al.*, 1991).

The uptake of heavy metals by plants and animals leads to their concentration in the food chains; posing a serious threat to human health.

Cadmium (Cd) is a very toxic heavy metal. The industrial uses of Cd are widespread and are increasing in electroplating, paint pigments, plastics, and silver-cadmium and nickel-cadmium batteries. A disease known as "Itai-Itai" in Japan is specifically associated with Cd poisoning, resulting in multiple bone fractures arising from osteomalacia (Crueger, 1984). Cadmium can also accumulate in the human body, and it has a half-life exceeding 10 years. There is evidence that low-level exposure to Cd, derived from the diet, is associated with renal dysfunction (Buchet *et al.*, 1990). Cd exposure has also been linked with pulmonary emphysema (Ryan *et al.*, 1982) and possibly bone demineralization (Bhattacharyya *et al.*, 1988).

Copper (Cu) also is a widespread industrial contaminant with toxic effects at many trophic levels. There are numerous industrial and manufacturing uses of this common metal. As a result, there are numerous sources of industrial Cu contamination. Absorption of excess copper by humans results in "Wilson disease" in which excess copper is deposited in the brain, skin, liver, pancreas, and myocardium (Harris, et al., 1996; Multhaup, et al., 1996).

Lead (Pb) is a widespread non-point source contaminant of urban environments, chiefly coming from lead paint and leaded gasoline. A number of the Superfund sites in the U.S. include those resulting from broken lead acid batteries where lead has migrated into ground water. Lead poisoning of children has also been linked to contemporary earthenware glazed surfaces (Myers, 1944) as well as pigments of old paints (Richmond, 1986). The toxicology of lead has been extensively studied. Inorganic lead (Pb^{2+}) is a general metabolic poison and enzyme inhibitor (like most of the heavy metals). Organic lead, in the form of tetra ethyl lead (TEL) or tetra methyl lead (TML), is even more poisonous than inorganic lead. The earliest symptoms of lead poisoning seem to be psychological (e.g., excitement, depression, and irritability). Young children are particularly affected and can suffer mental retardation and semipermanent brain damage. One of the most insidious effects of inorganic lead poisoning is its ability to replace calcium in bones, and to remain to form a semipermanent reservoir for long-term release well after initial absorption. The usual indicator of the degree of lead poisoning in humans is its content in whole blood. The disturbing fact is that present levels of lead in human blood (0.2 - 0.8 ppm) are already very close to that which is considered to be a reasonable toxicological limit, not leaving any margin for further exposure to lead (Aksu et al., 1990).

The removal of toxic and heavy metal contaminants from aqueous waste streams is one of the most important environmental problems in need of an effective and affordable solution. Traditional methods of sequestering heavy metals involve chemical engineering approaches in which the elements are precipitated or sorbed from the medium, such as chemical precipitation, ion exchange, and solvent extraction (Salt, *et al.*, 1995; Logan and Traina, 1993). These

methods are rarely selective and result in large volumes of waste in which the element of concern may still be a trace constituent. Selective removal of trace concentrations (mg/l to ug/l) of dissolved heavy metals from contaminated water and wastewater is a challenge and a commonly encountered problem facing various industries and publicly owned treatment works. The use of beads or granules of chemically stable polymeric chelating exchangers in fixed-bed processes has, to a great extent, resolved the problem but they are often found to be too expensive to justify their applications for heavy metal removal from water and wastewater (Logan and Traina, 1993).

The use of heavy metal-accumulating plants to remove toxic metals, including Cd, from soil and aqueous streams has been proposed as a possible solution to this problem (Wagner, 1994; reviewed by Salt *et al.*, 1995). Also a variety of biological materials nonspecifically bind heavy metals via charged groups (carboxylic acid, amino and sulfate groups) and there are proteins (metallothioneins) that selectively bind heavy metals via thiolate bonds (Schulze and Brand, 1978; Lepp, 1981; Robinson and Jackson, 1986; Steffens *et al.*, 1986; Butt and Ecker, 1987; Reese and Wagner, 1987; Kagi and Schaffer, 1988; Kuyucak and Volesky, 1989; Mahan *et al.*, 1989; Silver *et al.*, 1989; Rauser, 1990; Ke *et al.*, 1992).

The non-specific binding of heavy metals, particularly by cell wall fractions of algae including *Chlamydomonas reinhardtii*, has been exploited as a means to remove heavy metals from waste water (Mahan, *et al.*, 1989). In these studies, several different cell wall constituents have been implicated in metal binding including amine and carboxyl groups from both amino acids and polysaccharides, as well as sulfhydryl groups. Cadmium is a particularly favorable target metal for this new technology because it is readily transported and accumulated in several plants and algal species (Wagner, 1994).

The present invention is thus concerned with the removal of toxic metals as might be applied to industrial water effluent, ground water, natural surface water flows, and fresh and salt water bodies.

Metallothionein

Metallothionein is a general name given to heavy metal (Cd, Zn, Cu)-binding proteins having similar properties. They typically have low molecular weights, high contents of cysteinyl residues, and bind heavy metals tightly through mercaptide bonds.

Metallothioneins (MTs) were discovered in 1957 when Margoshes and Vallee searched for a tissue component responsible for the natural accumulation of cadmium in mammalian kidney (Margoshes and Vallee 1957). Metallothioneins are still the only biological compounds known to specifically bind this metal. However, as documented already in the earliest reports, cadmium is but one of the several metallic components bound by MTs, the others being most commonly zinc and copper. Since the discovery of MTs, intense interest in this small, structurally unique, and functionally enigmatic group of proteins has been driven by its putative roles in cadmium detoxification and essential metal metabolism.

Definition and Occurrence

The first MTs characterized were lower molecular weight, sulfur containing proteins, which consist of a single polypeptide chain of 61 amino acids residues. Twenty of the residues are cysteines which chelate up to seven bivalent cations, leaving neither free thiol groups nor a disulphide bridge (Kojima, *et al.*, 1976). Typically MTs also lack aromatic amino acids residues. All cysteines in MTs occur in the reduced form and are coordinated to the metal ions through mercaptide bonds, giving rise to spectroscopic features characteristic of metal-thiolate clusters (Martin, *et al.*, 1992).

In view of these unique chemical characteristics, the phenomenological definition was adopted that any polypeptide resembling mammalian MT in several of these features can be named an MT (Fowler *et al.*, 1987).

MTs now have been identified not only in the animal kingdom but also in higher plants, eukaryotic microorganisms, and in some prokaryotes (cited in Hamer 1986, Kagi and Kojima, 1987). To date, all organisms that have been screened contain one or more types of cysteine-rich polypeptides which selectively sequester heavy or soft metals including copper, zinc, lead,

cadmium, cobalt, nickel, mercury, silver and gold (Grill *et al.*, 1985; Jackson *et al.*, 1987; Kagi and Schaffer, 1988; Rauser, 1990).

Taking into account structural relationships, they can be subdivided into three classes (Fowler *et al.*, 1987, Furey *et al.*, 1986 Jackson *et al.*, 1987; Rauser, 1990). Class I MTs (MT-I) include mammalian MTs and polypeptides with related primary structure from other phyla. They are characterized by (1) molecular weight of about 10 kD (equivalent to about 60 amino residues), (2) the presence of 20 invariant cysteine (Cys) residues arranged in CysXCys clusters, where X is any amino acid, (3) the absence of aromatic amino acid residues, and (4) the presence of 6-7 metal-thiolate clusters whose number varies with respect to the type of metal bound. Class II MTs (MT-II) are low molecular weight and Cys-rich metal binding proteins, but the distribution of Cys residues does not correspond to that in mammalian MTs. These proteins have been identified in sea urchin, wheat, yeast, and certain prokaryotes (Kagi, 1993). The third class of MTs (MT-III) are nontranslationally synthesized metallothiolate polypeptides related to glutathione and have repetitive sequences: $(\gamma\text{-GluCys})_n\text{Gly}$, where n =2-11 (Rauser, 1990). The MT-IIIs are also known as phytochelatins (PC), and have smaller molecular weights (1-2 kD) than MT-I or MT-IIs. Phytochelatins, as their name implies, are found in plants.

Primary Structure of MTs

Owing to improvements in protein sequencing techniques and the facile determination of nucleotide sequences, primary structure data are now available for some class I MTs, class II MTs and various homologous sets of class III MTs (Rauser, 1990; Kagi, *et al.*, 1988). Table 1 shows some sequences of the three classes of MTs (Kagi, *et al.*, 1988). All class I and class II MTs characterized thus far are single-chain proteins.

Table 1. Classification of Metallothioneins and Amino Acid Sequences of Representative Forms.

Table 1.

Class I		
Human	MDP - NCSCAAGDSCTCAGS CKCKECTKCTS CKKSCCSCCBVG-CAKCAQGCI -CKJACD KCSCCA	
Chicken	MDPQDCTCAAGGSCSCAGS CKCKMCR -CRSCRKSCCSCCPAG - CNNCAKGCVCKEPASSKCSCCH	
Trout	MDP - - CECSKTGS CNCGGSCKCSN CA -CTSCKKS CCPCCPSD -CK- CASGCVCKGKTC DTS CCQ	
Crab	PDP- - - C -C - -NDKCDSKEGECKTG-CK- CTSCRCPPCEQCSSG - -C K- CANKEGCRKTCSKPCSCCP	
N. crassa	--GDCGC SGASS- - CNCGSG -CS - - - CSMCGSK	
Class II		
Sea urchin	MPDVKCVCCTEGKECACFGQDCCVTGECCKDGTCCGICINAACKCANGCKCGSGCSCTEGNCA	
Yeast	QNEGHECQCQCGSCKMNEQCKKSCSCPTGCNSDDKCPCGMKCEETKKSCCSGK	
Wheat	GCNDKCGCAVPCPGGTGCRCTSARCGAAAGEHTTCGCGEHCGGNPCACGGGEGTPSGCAN	
Synechocystis	TSTTLVKCACEPCLCNVDPSKAIDRNGLYYCCEACADGHTGGKGCGHTGCNC	
Class III		
S. pombe	ECECECG	
R. canina	ECECECECECECECECG	
P. vulgaris	ECECECECECECEC-b-alanine	

Mammalian forms contain 61-62 amino acid residues; chicken MT and sea urchin MTs contain 63-64 residues, respectively. Shorter single chain proteins are found in invertebrates and in certain fungi, the shortest one with 25 residues in *Neurospora crassa* (Lerch, 1980). The basic features of MT structure have been established by chemical and spectroscopic methods. The formation of two distinct metal clusters in rabbit liver MT was first shown by ^{113}Cd NMR (Otvos, *et al.*, 1980). The seven Cd^{2+} ions are bound in clusters of four and three metals formed by bridging and terminal cysteine thiolate ligands. All 20 cysteines participate in metal binding, and each of the seven metals is tetrahedrally bound. Rat liver MT can be cleaved by trypsin into two domains which bind metals individually in the same way as in the intact protein (Kagi, 1993). The N-terminal a-domain includes residues 1-30, which contain nine cysteines and form a cluster with three Cd or Zn liganded by three bridging and six terminal cysteine

ligands; the C-terminal b-domain contains residues 31-61, contains 11 cysteines and forms a cluster with four Cd or Zn liganded by five bridging and six terminal cysteine ligands (Fig. 1). The structure of MT in solution has been studied in detail by two dimensional ^{113}Cd-^{1}H NMR methods, and also confirmed by X-ray diffraction (Robbin, *et al.*, 1991). Three dimensional structures have been determined for rabbit liver Cd_7-MT, rat liver Cd_7-MT, and human liver Cd_7-MT (Kagi, 1993). These investigations have established the cysteine ligand to metal coordination pattern for the two domain clusters, they all show very similar conformations.

g-Glutamylcysteinylglycones detected in yeast, algae and certain plants are classified as class III MTs, or phytochelatins. The class III MTs are quite different in origin and structure although they show many phenomenological and functional similarities. Like the MTs of the higher organisms they are isolated from the cell cytosol as low-molecular weight, metal rich complexes. They are synthesized by enzymatic reactions rather than on ribosomes. Their structures involve unusual repeating sequences of g-Glu linkages to cysteine, which produce the general formula: g(-Glu-Cys)$_n$ Gly {(g-EC)$_n$G} (Fig. 2). The n value typically ranges from 2-8 or higher and sometimes Gly is replaced by b-Ala, Ser or Glu. The major sources of peptides for structural analysis have been Cd-binding complexes from *S. pombe* and various plants, particularly tissue culture cells (Rauser 1993). The structure of (g-EC)$_n$G peptide was confirmed by chemical synthesis of penta-and heptapepides (Kondo *et al.*, 1985) and the nanopeptide (Grill *et al.*, 1985). The recurrent g-carboxyamide linkage prevented exclusive use of Edman protein sequencing methodology. Steffens *et al.*, (1986) confirmed the sequence of the hepta- and nanopeptides from tomato (*Lycopersicon esculentum*) by mass spectrometry. Enzymatic digestion provided the sequence information of peptides from *Datura innoxia*, the g-glutamyl linkages were identified by ^{113}Cd-NMR spectroscopy (Jackson *et al.*, 1987). Using Cd as the inducing metal, the presence of these peptides (g-EC)$_n$G was tested in three dicotyledonous plants, the alga *Chlorella fusca,* and other species (Gekeler *et al.*, 1988). A small amount of (g-EC)$_2$G occurred in *Neurospora crassa.* The n = 2 to 6 peptides were also

detected in six species of Basidiomycetes and four species of *Schizosaccharomyces* in addition to *S. pombe.*

The three classes of MTs are different in their affinities for heavy metals. The binding constants for cadmium range from $K_3=10^{25.5}$ M^{-1} for equine MT-I to $K_3=10^{19}$ M^{-1} for tobacco MT-II (Rauser, 1990). Another measure of metal binding affinity is the pH at which 50% of the bound metal is released from the protein. The higher the affinity for the metal, the lower the pH that is necessary to displace the metal from the protein. Typically, metal ions are displaced from MTs at pH values ranging from 2.0 - 4.5, with copper and cadmium generally being released at lower pH values. The Cd-MT from cabbage lost half its Cd at pH 4.4 (Wagner, 1984), the complex from tobacco did so at pH 5-5.8, whereas Cd-MT from rat liver dissociated by 50% at pH 3 (Reese, 1987). Kagi and Vallee (1988) found pH values of 3 and 4.5 for Cd and Zn dissociation, respectively, for equine Cd, Zn-MT. The pH-dependent selective release of heavy metals can also be used to selectively harvest heavy metals that vary with respect to their binding constants (Kagi and Schaffer, 1988; Rauser, 1990).

Biological Functions

Metallothioneins are an unusual group of proteins or polypeptides which have challenged the interest of chemists and life scientists alike for over 30 years. In spite of the rich information on its structure, MT is a protein in search of functions (Karin 1985). More than three decades after the discovery of MT, its functional significance remains a topic of discussion (Karin, 1985: Bremner, 1987). Questions remain with respect to how cadmium exerts its toxicity and its mechanism for exercising its defensive action. The conservation of the structure, the ubiquitous occurrence, and the programmed synthesis of MT in regeneration and development are strong arguments for its playing a crucial role in some fundamental metal-related cell biological process. The main hypotheses thus far considered are that: (1) MT serves as a relatively non-specified metal-buffering ligand to either sequester or dispense metal ions, or

(2) it has a specialized function in normal cellular metabolism or development (Kagi and Schaffer, 1988). It may also serve a number of different biological purposes.

That MT is the cellular component responsible for much of the intracellular sequestration of Cd, bringing about the long biological half-life of this nonessential element, is unquestioned (Webb, 1987a). Through gene amplification (Griffith *et al.*, 1983, Durnam and Palmiter 1987) and transfer (Thiele *et al.* 1986, Ecker *et al.* 1986) experiments, the presence of MT is shown to be strongly associated with Cd detoxification. In cultured mammalian cell lines stable resistance to Cd can be brought about by massive amplification of the MT genes (Beach, *et al.*, 1981). However, the production of excessive amounts of Cd-containing MT has been suggested as a causative factor in bringing about kidney damage in chronic Cd poisoning (Nordberg, *et al.*, 1987), thus causing doubt on the biological importance of MT synthesis as a specific and effective Cd defense mechanism in animals. MTs have also been implicated in the sequestration of other nonessential metals, such as Hg, Ag, Au, Pt and Pb (Webb, *et al.*, 1987a). Such effects have been claimed to be responsible for the development of resistance toward Au- and Pt-containing drugs in cultured cells and the selective protection of some tissue from such agents in animals following preinduction of MT (Naganuma *et al.*, 1987; Monia *et al.*, 1987).

Hypersensitivity to elevated trace-metal concentrations has also been observed in fungal (Hamer, 1986) and prokaryotic (Turner, et. al., 1993) cells with deleted MT genes, selected for enhanced tolerance to certain trace metal ions. *Saccharomyces cerevisiae* cells lacking the MT gene (CUP1) have normal cell growth, differentiation, and copper metabolism. These mutants grow with normal doubling times in standard, low-copper media, and are capable of mating, diplophase growth, germination and accumulation of copper (cited in Hamer, 1986). CUP1-deficient cells are, however, hypersensitive to elevated concentrations of copper. Sequestration of excess copper may be an exclusive role of MT in *Saccharomyces cerevisiae*.

The preponderance of Zn in most MT preparations and the responsiveness of MT-bound Zn and Cu to the dietary supplies of these essential nutrients suggest a role in their metabolism and have led to a large number of studies (cited in Bremner 1987). As a homeostatic mediator,

MT could donate metal ions in the biosynthesis of Zn and Cu-containing metalloenzymes and metalloproteins (Brady, 1982). The emergence of Cu-MT in *N. Crassa* prior to the formation of the Cu-containing enzymes would be in concert with such a role (Huber *et al.*, 1987), as are *in vitro* experiments which have demonstrated that Cu and Zn can be transferred from MT to the apo forms of a number of Cu and Zn proteins (Beltramini, *et al*, 1982, Brady, 1982). Conversely, when Cu and Zn accumulated intracellularly, the reactive ions can be sequestered in a chemically innocuous form by binding to newly synthesized apoMT. This mechanism is believed to account for the accumulation of large amounts of Cu-MT in tissues and cells of organisms affected with an inherited disorder of Cu metabolism (Sternlieb, 1987). Protection from the effects of excessive ionic Cu by sequestration is thus far the only documented benefit of MT induction in yeast (Thiele, *et al.*, 1986; Ecker *et al.*, 1986), *N. Crassa* (Lerch *et al.*, 1980), and in copper-resistant forms of *Agrostis gigantea* (Rauser, *et al.*, 1980).

A biological role, probably unrelated to a detoxification function for metals, is suggested by the fact that in certain tissues and cell types MT is induced by chemical and physical stress. These effects, which are most prominent in liver and mediated in part by hormones, resemble an acute phase response (Bremner, 1987). In some cases, such as in the exposure to electrophilic agents, i.e., O_2, free radicals, and alkylating agents, the increased supply of MT could provide neutralizing nucleophilic equivalents. However, in most other instances it is unclear how the organism benefits from increased MT biosynthesis.

One possibility is that MTs may have metalloregulation function in cellular repair processes, growth, and differentiation. This was first suggested by the parallelism of enhanced RNA synthesis with increased Zn-MT formation observed in the liver of rats recovering from partial hepatectomy (Ohtake *et al.*, 1978) and is supported by the programmed regulation of MT mRNA and protein levels during embryogenesis (Nemer *et al.*, 1984) and at different stages of fetal and prenatal development (Andrews, *et al.*, 1984). In view of the known effects of Zn on embryogenesis, its participation in RNA polymerases and its serving as a structural modulator

of the Zn finger domain in several DNA-binding proteins, it is tempting to hypothesize that Zn-MT plays a part in expression of genetic information.

Table 2. Factors that Induce Metallothionein Synthesis in Cultured or *in vivo* Animal Cells.

Table 2.

metal ions: Cd, Zn, Cu, Hg, Au, Ag, Co, Ni, Bi.	Streptozotocin
glucocorticoids	2-propanol
progesterone	ethanol
nitrogen	ethionine
glucagon	alkylating agents
catecholamines	chloroform
interleukin I	starvation
interferon	infection
endotoxin	physical stress
dextran	X-irradiation
	high O_2 tesion

for citations, see Palmiter (1987) and Bremner (1987).

Molecular Biology of MTs

While all plants examined to data contain phytochelatins, apparently, only a few contain class I and class II MTs. An MT-I has been identified in immature embryos of wheat and MT-I has been identified in barley, pea, *Mimmulus sp.*, *Synechoccocus.sp.*, maize, and soybean (de Miranda *et al.*, 1990; Kawashima *et al.*, 1991, 1992; Lindow *et al.*, 1989; Reese & Wager, 1987; Robinson *et al.*, 1992; Shimizu *et al*; 1992; Steffens *et al.*, 1986). During the past years, different MT cDNAs have been cloned from eukaryotic cells (Giffith, *et al.*, 1983; Peterson *et al.*, 1984; Schmidt and Hamer, 1983; Thiele *et al*, 1986). Genes encoding MTs from one organism have also been transferred and expressed in other organisms. Plant and animal class I

and II MTs have been expressed in *E.coli* and in transgenic plants (tobacco, *Brassica campestris* and *Arabidopsis thaliana*, Evans *et al.*, 1992; Lefebvre *et al.*, 1987; Maiti *et al.*, 1989, Odawara *et al.*, 1995). Significantly, the expression of foreign MTs increased the heavy metal binding capacity of each host. *E.coli* cells expressing human MT-I fusion proteins bound 66-fold more Cd than non-MT-I expressing cells and accumulated 90% of the total Cd present in the medium (2.0 uM Cd in medium) (Jacobs *et al.*, 1989). Transgenic *Brassica campestris* plants expressing the Chinese hamster MT-II had a four-fold increase in Cd binding capacity (Lefebvre *et al.*, 1987). Furthermore all the Cd in the plants (grown in 1.0 mM Cd) was bound to MT-II. Similar studies with transgenic tobacco and *Arabidopsis* plants expressing either a mouse or pea MT-II, respectively, demonstrated that transgenic plants had a higher resistance to Cd poisoning (Evens *et al.*, 1992; Maiti *et al*; 1989). While these studies indicate that expression of class I and II MTs in transgenic organisms can increase their heavy metal binding capacity, there is evidence that elevated expression of phytochelatin (class III MTs) does not increase heavy metal tolerance (Harmens *et al.*, 1993). As indicated earlier, the heavy metal binding constant of phytochelatins is seven orders of magnitude lower than those of class I and II MTs. These results indicate that the expression of higher binding affinity MTs (class I and II) is likely to increase heavy metal binding capacity unlike the expression of phytochelatins.

Objectives

The use of algal cells for the removal and/or detoxification of pollutants from waste-water has been the subject of a number of studies in recent years. Reports include those on the removal of phosphate, ammonium (Chevalier, *et al.*, 1985); copper (Darnall, *et al.*, 1986); mercury (Pant *et al.*, 1992); nitrate, nitrite, chlorinated hydrocarbons (Pore *et al.*, 1984); uranium, gold, silver, and a variety of other heavy metals (Nakajima *et al.*, 1982).

Unicellular green algae, some species of which are amenable to genetic analysis, have often been used as experimental models for the study of basic biochemical processes. Several

green algal species have been reported to accumulate Cd-binding components upon exposure to cadmium (Gekeler, *et al.*, 1988; Weber, *et al.*, 1987). Recent characterization of these components from a wide range of algal species has shown them to be phytochelatins (Gekeler, *et al.*, 1988).

Many of the features of the biology of algae make them potential candidates for the treatment of heavy metal contamination water. Unlike animals, algae are tolerant of high levels of heavy metals in their growth medium (Howe, *et al*, 1992); they can grow autotrophically and hence presumably retain biocatalytic activity; they have large ratios of surface area to volume; they are easy to harvest, and they have the potential for genetic manipulation (Roffey *et al.*, 1991). Also algae can replace many copper-containing proteins under copper depression with heme containing proteins (Howe and Merchant, 1992). Thus Cu sequestration by MTs may not be detrimental to algal metabolism.

Summary of the Invention

In the present invention, the capacity of the green alga, *C. reinhardtii,* to produce the metal-binding factors, in response to stress induced by the heavy metal Cd (Howe *et al.* 1992) has been addressed. Gekeler and coworkers (1988) reported that *C. reinhardtii* accumulates phytochelatins, with n values range from 2 to 4, in response to Cd- induced stress. Howe *et al.* (1992) extended this observation by showing that newly synthesized Cd-induced peptides assemble into Cd-binding peptide complexes that chelate up to 70% of the intracellular Cd ions in extracts of Cd-treated cells.

The current view of heavy metal detoxification by phytochelatin is that the process consists of at least two steps. The first is the activation of phytochelatin synthase as a result of

an increase in the intracellular concentration of heavy metal. The second step in the detoxification response is the chelation of heavy metals by the newly synthesized phytochelatins. This second step is presumably directly responsible for heavy metal tolerance, because, by binding to metals with a relatively high affinity, phytochelatins prevent toxic reactions between heavy metals and essential macromolecules, e.g. enzymes, nucleic acids, lipids. A proposal by Vogeli-Lange and Wagner (1990) suggested that phytochelatins mediate metal detoxification owing not merely to their function as cytoplasmic metal chelators but also by serving to transport heavy metals from the cytoplasm to the vacuole.

As described previously, the MTs commonly found in higher plants (phytochelatins) have the lowest binding affinities for heavy metals whereas those commonly found in mammals have the highest binding affinities. An object of the present invention is to enhance the heavy metal binding capacity of algae by expressing a chicken MT-I gene in *Chlamydomonas*. The chicken MT-I gene was fused to the 5' promoter of the *Chlamydomonas* b_2-tubulin gene and the 3' terminator of the arylsulfatase gene. This construction was co-transformed with a separate plasmid (p389) containing the nuclear encoded argininosuccinate lyase. The host strain is CC-425 (arg-2 lesion) which is unable to grow in the absence of arginine (Davies *et al.*, 1992) but which is complemented by p389. Molecular studies indicate that the expression of genes encoding the MT-I and MT-II as well as the enzymes which synthesize MT-III are induced following exposure to heavy metals (Jackson *et al.*, 1987; de Miranda *et al.*, 1990; Robinson *et al.*, 1986; Scheller *et al.*, 1987; Steffen *et al.*, 1986). As it is not known whether such heterologous regulatory signals operate in *Chlamydomonas*, the MT was expressed under the regulation of an inducible b_2-tubulin promoter (Davies, *et al.*, 1992). Typically, transcription

is induced several fold from the b_2-tubulin promoter following de-flagellation of the cells (Davies, *et al.*, 1992). Co-transformed cells which grow on arginine were screened for the presence of the MT gene by Southern blot analysis. The level of expression of the MT gene was also determined in various transformants by northern blot analysis. Recombinant algae expressing chicken MT-I were then compared to wild type algae for heavy metal binding capacity, and will be used to sequester heavy metals from contaminated water and soil.

Thus, some of the objects of the present invention include the generation of transgenic algae expressing high affinity heavy metal binding proteins. The present invention specifically allows for the determination of the tolerance of *Chlamydomonas* for cadmium, and the characterization of the heavy metal binding sites on unicellular algae. The present invention also allows for the comparison of the heavy metal binding capacity of wild type and transformed algae.

Another problem addressed by the present invention is the selective separation of metals, particularly the separation of precious and desirable metals, such as gold and uranium, from other metals such as cadmium, zinc and copper. Such problems are faced in the mining industry where current technology involves the use of toxic substances (such as cyanide) that pose a pollution threat to the environment where mining operations take place.

Accordingly, it is another object of the present invention to facilitate the selective recovery of such precious and rare metals from mineral sources where aqueous media can be used, such as in natural surface water flows, ground water and where water may be introduced.

The present invention thus includes a transgenic algal cell and a method of its production. The present invention also includes methods involving the use of such transgenic algal cells.

In general terms, the transgenic algal cell of the present invention comprises: a transgenic algal cell of the genus Chlamydomonas and comprises reproductive genetic material comprising a nucleotide sequence, the nucleotide sequence capable of expressing chicken Type I Metallothionein. Preferably, the transgenic algal cell of the present invention is from the strain *Chlamydomonas reinhardtii*. The transgenic plant cell of the present invention may include transgenic algal cells selected from the strains of Chlamydomonas viable in marine environments and the strains of Chlamydomonas viable in frozen environments. It is preferred that the transgenic algal cells be in a dried state.

The present invention also includes a method of removing metal from an aqueous medium containing at least one dissolved or suspended metals, the method comprising the steps: (1) bringing into contact with the aqueous medium transgenic algal cells of the genus Chlamydomonas comprising reproductive genetic material comprising a nucleotide sequence, the nucleotide sequence capable of expressing the metal-binding protein chicken Type I Metallothionein in the transgenic algal cells; (2) maintaining the transgenic algal cells in the aqueous medium so as to allow at least one metal to bind to the metal-binding protein, so as to produce a metal-bound adduct of the metal-binding protein; and (3) removing the transgenic algal cells from the aqueous medium.

The transgenic algal cells used in accordance with this method of the present invention may be from the strain Chlamydomonas reinhardtii, such as those selected from species of

Chlamydomonas viable in marine environments and species of Chlamydomonas viable in frozen environments. The transgenic algal cells may be in the form of living cells viable in the aqueous medium, and may be in a dried state prior to introduction into the aqueous medium.

The method of the present invention may be used to remove a wide variety of metals, such as one or more of those selected from the group consisting of cadmium, copper, zinc, gold and uranium.

In an alternative embodiment, which is preferred, the aqueous medium in step 3 has an initial pH; and the method also involves the step of (4) altering the pH of the transgenic algal cells removed from the aqueous medium in step 3 to a lower pH so as to cause the at least one metal to be released from the metal-binding protein, so as to regenerate the metal-binding protein.

For instance, the initial pH value may be about 7 and the lower pH value may be brought to about 2.

The method of the present invention additionally may comprise repeating steps 1 through 3 with the metal-binding protein regenerated in step 4.

In one embodiment, the aqueous medium may be below ground and the transgenic algal cells may be immobilized below ground during steps 1 and 2, and brought above ground prior to carrying out step 3. In another variation, the aqueous medium may be below ground and the transgenic algal cells may be immobilized below ground during steps 1 and 2, and brought above ground prior to carrying out steps 3 and 4.

The present invention also includes a method of separating a first metal selected from the group consisting of gold or uranium, and mixtures thereof, from at least one second metal,

in an aqueous medium, the method comprising the steps: (1) bringing into contact with the aqueous medium transgenic algal cells of the genus Chlamydomonas comprising reproductive genetic material comprising a nucleotide sequence, the nucleotide sequence capable of expressing the metal-binding protein chicken Type I Metallothionein in the transgenic algal cells; (2) maintaining the transgenic algal cells in the aqueous medium at an initial pH so as to allow the first metal and at least one second metal to bind to the metal-binding protein, so as to produce a metal-bound adduct of the metal-binding protein comprising both the first metal and the at least one second metal; (3) altering the initial pH of the transgenic algal cells to a lower pH so as to cause the at least one second metal to be released into a solution from the metal-bound adduct of metal-binding protein; and (4) separating the transgenic algal cells from the solution containing at least one second metal.

The transgenic algal cells used in accordance with this method of the present invention may be from the strain Chlamydomonas reinhardtii, such as those selected from species of Chlamydomonas viable in marine environments and species of Chlamydomonas viable in frozen environments. The transgenic algal cells may be in the form of living cells viable in the aqueous medium, and may be in a dried state prior to introduction into the aqueous medium.

This method of the present invention may involve variations wherein the second metal(s) is/are selected from the group consisting of cadmium, copper and zinc.

Typically, the initial pH will be about 7, but may vary depending upon conditions, or be varied through active buffering, etc.

In one embodiment, this method of the present invention may additionally comprise the step of: (5) further lowering the pH of the transgenic algal cells removed from the aqueous

medium in step 4 to a still lower pH so as to cause the first metal to be released from the metal-binding protein, so as to regenerate the metal-binding protein. Typically, the pH to which the transgenic algal cells are lowered in step 5 is below 3.

This method may also involve the variation of repeating steps 1 through 3 with the metal-binding protein regenerated in step 5.

In another optional variation, this method may be carried out with the aqueous medium being below ground and wherein the transgenic algal cells are immobilized below ground during steps 1 and 2, and then brought above ground prior to carrying out step 3. Likewise, the aqueous medium may be below ground and the transgenic algal cells immobilized below ground during steps 1 and 2, and then brought above ground prior to carrying out steps 3 and 4.

The compositions of the present invention may be used, and the methods of the present invention may be carried out, using known laboratory and industrial equipment, set-ups, devices, and plant arrangements, such as are known in the fields of chemistry, chemical engineering, the well-drilling and soil and water remediation arts, the mining field, and industrial engineering. The methods of the present invention thus may be scaled up or down as appropriate, and carried out in such applications as in plant or waste water facility environments, in the field for soil and water remediation, or for mining purposes.

The present invention also includes a method for producing transformed algal cells that contain and express a class I mammalian metallothionein gene (MT-I) comprising the steps of: (1) excising an MT-I gene from a plasmid containing an MT-I gene; (2) generating cloning sites on a plasmid encoding a promoter and a translation termination site; (3) amplifying the DNA in the region between the promoter gene and the termination site; (4) cloning the MT-I gene into

the plasmid containing the amplified DNA to produce a recombinant plasmid; (5) ligating the MT-I gene into the recombinant plasmid at the cloning sites; and (6) transforming the algal cells with the recombinant plasmid containing the MT-I gene. The method of the present invention may be used on any of the algal strains mentioned herein including those of the genus *Chlamydomonas.*

Finally, the present invention includes a transgenic algae produced by this method.

Brief Description of the Drawings

Figure 1 shows a schematic of a sequence for Rabbit Liver MT-II Showing Connectivities for the Cd-S Bond in Cd_7-MT. (A) amino acids that are part of a-domain, (B) amino acids that are part of the b-domain. The large dark ovals represent cysteine residues (Stillman, et al., 1992).

Figure 2 shows the structure of various g-Glu-Cys Peptides. The α-carboxy carbon of Glu is highlighted in black in panel A to differentiate between α- and γ-carboxy-amide linkages. B is a model summarizing the five families of γ-Glu-Cys peptides involved in metal sequestration in plants and certain yeasts. The lines connecting peptides refers to family relationships and does not necessarily specify biosynthetic sequence. Adapted from Meuwly *et al.* (1995).

Figure 3 shows a schematic of plasmid pMTCC containing Chicken MT-I. MT-I was excised from pMT6c7 by enzyme digestion with Hind III and EcoR I. Enzyme restriction sites Hind III and EcoR I were generated for transformation vector pJD55 by PCR. MT-I was cloned into that PCR generated plasmid.

Figure 4 is a graph showing growth of *Chlamydomonas* Cells (CC-2137) as a Function of Cadmium Concentration. {□}, no additions; {☞} 1 uM $CdCl_2$; {Δ} 5 um$CdCl_2$; {☆} 10 uM $CdCl_2$; {hexagon} 20 uM $CdCl_2$; {✧} 40 uM $CdCl_2$. Data are the average of three separate experiments.

Figure 5 is a graph showing predicted Chemical Activity of Cadmium in TAP Medium. pCd^{2+} is - log of Cd Activity.

Figure 6 is a graph showing growth of *Chlamydomonas* Cells in the presence and absence of Cd and Glutathione Synthesis Inhibitor BSO. {□} No addition; {O} 5 um BSO; {Δ} 10 um BSO; {∇} 25 uM BSO; {☆} 50 uM BSO; {✧} 100 uM BSO; {■} 40 uM $CdCd_2$; {●} 40 uM $CdCl_2$ plus 5 uM BSO; {♦} 40 uM $CdCl_2$ plus 10 uM BSO; {∀} 40 uM $CdCl_2$ plus 25 uM BSO; {✱} 40 uM $CdCl_2$ plus 50 uM BSO; {◆} 40 uM $CdCl_2$ plus 100 uM BSO. Data are the average of three separate experiments.

Figure 7 shows an ethidium bromide stained gel of plasmid pMTCC digested with Hind III and EcoR I. The arrows indicate the positions and sizes of the fragments. Lane 1: 1 kbp ladder; Lane 2, 3, 4, 5 are the restricted pMTCC with Hind III and EcoR I. About 2 ug of DNA was loaded into each lane.

Figure 8 shows a southern blot analysis for cloned pMTCC. Lane 1: pMTCC was digested with Kpn I and Sca I. Lane 2: Chicken MT-I fragment (positive control). About 300 ng of DNA was loaded into each lane. Probed with 300 bp Chicken MT-I gene.

Figure 9 shows a southern blot analysis: Lane 1: the chicken MT-I fragment (positive control); Lane 2: plasmid pMTCC; Lane 3: control CC-425; Lane 4: transformant. The arrows

indicate the positions and sizes of hybridizing fragments. About 1 ug of DNA was loaded on each lane and 25 ng of MT-II DNA was used as probe.

Figure 10 shows a northern blot probed with ^{32}P-Labeled MT-II DNA. Lane 1 and Lane 2 are the same transformants pMTCC4; Lane 3 is transformant pMTCC8; Lane 4 is control strain CC-425; and Lane 5 to 8 are the deflagellated cells corresponding to the previous four lanes. 15 ug of total RNA was loaded into each lane.

Figure 11 is a graph showing cell growth curve for transformed and wild type (CC-425) *Chlamydomonas reinhardtii*. Date are average of three separate experiments.

Figure 12 is a graph showing growth of wild type (CC-425) and transformed (MT-II) (pMTCC4 and pMTCC8) *Chlamydomonas* in TAP plus 40 uM $CdCl_2$. Data are the average of three separate experiments. Error bars represent one standard deviation.

Figure 13 is a graph showing the removal of cadmium over time from an aqueous solution through the incremental addition of algal cells, in accordance with one embodiment of the present invention.

Figure 14 is a graph showing the pH dependency of metal binding to algal cells allowing for the regeneration of cells, in accordance with one embodiment of the present invention.

Figure 15 is a graph showing the binding of gold, uranium and cadmium by dried algal cells as a function of pH, in accordance with one embodiment of the present invention.

Figure 16 is a graph showing a comparison of the pH dependent cadmium binding properties of dried walled (normal) cells and dried cell wall-less cells at high ionic strength, in accordance with one embodiment of the present invention.

Figure 17 is a graph showing the removal of Cd from different solutions containing competing metal ions at pH 5.

Figure 18 is a graph showing the removal of metals from multimetal ion mixtures at pH 5.

Figure 19 is a graph showing the removal of metals from two-metal ion mixtures (Cd and another metal) at pH 5.

Figure 20 is a graph showing the removal of uranium from different metal ion mixtures at pH 5.

Figure 21 is a graph showing the removal of gold from different metal ion mixtures at pH 4.3.

Figure 22 is a graph showing the removal of Cd from different solutions containing competing metal ions at pH 5.

Figure 23 is a graph showing the removal of metals from multimetal ion mixtures at pH 5.

Figure 24 is a graph showing the removal of metals from two-metal ion mixtures (Cd and another metal) at pH 5.

Figure 25 is a graph showing the removal of uranium from different metal ion mixtures at pH 5.

Figure 26 is a graph showing the removal of gold from different metal ion mixtures at pH 4.3.

Detailed Description of the Preferred Embodiments

In accordance with the foregoing summary of the invention, the following presents a detailed description of one embodiment of the present invention which is presently considered to be the best mode of practicing the invention.

Materials and Methods

Chlamydomonas reinhardtii strains and DNAs

C. reinhardtii wild type strains CC-2137 and CC- 425 were used in this example. Plasmid pMT6c7 (3.4 Kb) which contains the chicken MT cDNA was kindly provided by Dr. Glen K. Andrews, Biochemistry and Molecular Biology Department at the University of Kansas Medical Center. Plasmid pJD55 (11 Kb) which encodes the b_2 - tubulin (b_2t) promoter and arylsulfatase (Ars) gene was kindly provided by Dr. John Davis Department of Plant Biology, Carnegie Institution of Washington, Stanford, CA). Plasmid p389 was provided from the *Chlamydomonas* Genetic Stock Center, Duke University, Durham, NC.

Cell Culture Growth and Extraction

Wild type *C. reinhardtii* cells (strain CC-2137) were grown in 250 milliliter Erlenmeyer flasks containing 100 milliliters of tris acetate phosphate (TAP) medium (Gorman, *et al.*, 1965). Strain CC-425 cell cultures were grown in TAP medium and supplemented with 50 ug/ml arginine (Harris, 1989). The cells were grown at 25°C under continuous illumination (80 uE/m^2/sec). The cell innoculum for each treatment was approximately 10^4 cells/ml. Cell growth was determined by counting the cells with a hemocytometer or by determination of chlorophyll content (Arnon, 1949). The cells were harvested by centrifugation (5,000 g for 5 min., at 4°C), then washed twice in a solution containing 50 mM potassium phosphate, pH 7.0, and centrifuged again. For determinations of cell growth response to Cd, the cell culture growth

was monitored for 4-6 days. For determinations of bound Cd in cell fractions, $CdCl_2$ was added to 1 L of walled cell culture (CC-2137) at the initiation of the growth study to give a final concentration of 40 uM. The cells were harvested at the end of five or six days, washed twice with 50 mM potassium phosphate buffer (pH 7.0), ruptured in a French Press at 4,000 psi, followed by ultracentrifugation at 100,000 X g for 2 hours at 4°C to separate the soluble fraction from cell wall and membrane fractions. Each fraction was digested with concentrated 2N nitric acid for Cd determinations by a Perkin-Elmer 403 atomic absorption spectrophotometer using an air-acetylene flame with single element lamps. Concentration of metals (Cd and Mg) was determined by comparing experimental samples with commercially available standards of 1000 ug/ml (Fisher, Cincinnati, OH) which were diluted to appropriate concentrations in double distilled water. The chemical activity of Cd in TAP was determined using the program SoilChem (Sposito and Coves, 1988).

For the heavy metal exchange studies of chlorophyll, cell strain CC-2137 was grown in 1 L TAP for 4 days, and 40 uM $CdCl_2$ was added either at the beginning or end of the cell growth. The cells were harvested at 4,000 g for 10 min., washed in 100 milliliters of TAP media, pelleted, extracted in 10 milliliters of 100% methanol by vortexing, and pelleted at 20,000 g for 10 min. The supernatant was filtered (0.2 micon), dried under vacuum, and then washed with 10 milliliters of water and dried again. The pellet was resuspended in 10 milliliters of methanol, and the metal (Cd and Mg) content was measured on an Atomic Absorption Spectrophotometer.

Plasmid Construction and Co-Transformation of *Chlamydomonas*

E coli. plasmids were purified in quantity by cesium chloride/ethidium bromide gradient centrifugation (Little, 1988). Small amount of plasmids (minilysates) for the screening purposes were prepared by the method of Maniatis, *et al.* (1982). For cloning of the chicken MT I gene into the transformation vector (pJD55), a 300 bp fragment containing the MT coding sequence was excised from the pMT6c7 plasmid by restriction digestion with Hind III and EcoR I (Fernando *et al.*, 1989). DNA was electrophoresed in a 0.8% agarose gel in TAE, and the MT fragment of approximately 300 bp was isolated from the gel using glass milk (Boyle and Lew, 1995).

PCR primer 1 and primer 2 comprising 31 bp and 34 bp, respectively, which corresponded exactly to regions within the 5' b_2 tubulin initiation site and 3'Ars translation termination site, were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Primer 1 (5'-TTAAGCTTTAATAAACACAAAAGCGATATCG-3') was designed to initiate extension from the b_2- tubulin gene towards the 3' terminus and primer 2 (5'AGCTTAAGCTTGATGGTTTCGTCCTGAGCCGGTG -3') from the *Ars* gene towards the 5'terminus. The 5' region of primers 2 and 1 contained Hind III and EcoR I restriction endonuclease recognition sites, respectively, to generate cloning sites on pJD55 plasmid which contained the b_2- tubulin promoter and Ars 3' untranslated terminator region.

PCR amplification was accomplished in a Perkin Elmer Cetus DNA thermal cycle and performed as described by Wayne M. Barnes (1994). The PCR reaction buffer consisted of 200 mM Tris-HCL (pH 8.55 at 25°C), bovine serum albumin at 150 ug/ml, 160 mM $(NH_4)_2SO_4$, 35 mM $MgCl_2$, and 2.5 mM of each dNTP. Each reaction solution contained 20 pmol of each primer, 0.1-10 ng of DNA template, and 5U of Vent DNA polymerase (New England Biolabs,

Inc., Beverly, MA). Reactions were made up to 100 ul, split into identical fractions of 33 ul, and overlayed with 40 ul of mineral oil. Prior to amplification the template DNA was denatured at 94°C for 1 min. The reaction consisted of 30 cycles of 30 sec at 94°C for denaturation, 30 sec at 55°C for primer annealing, and 5 min. at 72°C for primer extension, followed by a final extension period of 15 min. at 72°C. PCR products were electrophoresed in a 0.8% agarose gel in TAE, and DNA was isolated from the gel using glass milk as previously described. For further purification, DNA was precipitated by first adding 8.0 ul of 4 M NaCl, and then adding 40 ul of autoclaved 13% (w/v) PEG. After thoroughly mixing, the sample was incubated on ice for 20 min, and the DNA pelleted by centrifugation for 15 min. at 4°C. The pellet was then washed with 70% ethanol, dried under vacuum, and resuspended in 20 ul TE.

The chicken MT I gene was cloned into the pJD55 PCR fragment (about 5 Kb) Hind III and EcoR I sites (PCR product). Ligation of the fragments was performed according to Maniatis *et al.* (1982) in a 20 ul reaction mixture over night at 16 °C using 1 unit of T_4 DNA ligase. The DNA was then transformed into $RuCl_2$-treated *E.coli* competent cells (DH5 a). Cells carrying the recombinant plasmid were grown on Luria broth plates (10g/L tryptone; 10g/L NaCl; 5g/L yeast extract; 15 g/L agar) with 10 ng/ml IPTG, 4 ug/ml X-Gal and 100ug/ml ampicillin. Transformants were selected as ampicillin-resistant white colonies. The recombinant plasmid (named pMTCC) was prepared from positive colonies by the modification of an alkaline extraction procedure (Maniatis, *et al.*, 1982). The insertion of MT-I was confirmed by restriction enzyme digestion with Hind III and EcoR I, and also by Southern transfer of Kpn I and Sca I digested plasmid probed with the chicken MT gene (Figure 9). The entire construction of plasmid (pMTCC) is described in Figure 3.

C. reinhardtii strain CC-425 was co-transformed by the glass bead method of Kindle (1990) with plasmids p389, containing the argininosuccinate lyase gene which enables transformants of CC-425 to grow on arginine-deficient medium, and pMTCC, containing the MT-I chimeric gene. Glass beads, 0.45-0.52 mm in diameter, were obtained from Sigma (St. Louis, MO). They were acid (HCl) pre-washed, then rinsed thoroughly with distilled water, dried, and sterilized by autoclaving. 100 mililiters of CC-425 cells were grown to a density of 10^6 cells/ml in TAP medium plus 50 ug/ml arginine. The cells were then transferred to two 50 mililiters sterile tubes and pelleted at 2,000 xg for 2 min. The pellets were resuspended in 1 mililiter fresh media and shaken at room temperature for 2-4 hours. Glass beads (300mg) were added to a 1.5 mililiters microfuge tube which contained 0.3 mililiter of CC- 425 cells and 0.1 mililiter of 20% (w/v) PEG (Polyethylene glycol) and 1-2 ug DNA (1: 3 ratio (w/w) of p387 to pMTCC). The cells were then agitated at top speed on a votex mixer. The contents of the microfuge tubes were transferred into a culture tube containing 5 mililiters of non-selective media (TAP+50ug/ml arg) and shaken overnight to allow for DNA integration. The beads were allowed to settle, and cells were spread on selective agar plates (TAP plates) with a glass spreader. The plates were allowed to dry and sealed with Parafilm and incubated at room temperature. The transformed colonies were visible after 10-12 days.

Southern Blot Analysis

C. reinhardtii DNA from control (CC-425) and co-transformed strains was isolated by a modified Eluquik DNA purification procedure (Schleicher & Schuell, Keene, New Hampshire). Cells (1.6 mililiters) were pelleted in a microfuge tube by centrifugation at 7,000 g for 30 sec. The cells were then frozen in liquid N_2. Lysis buffer (80ul) was added immediately and the

tube was agitated until the suspension became clear and uniform. This was followed by adding 160 ul of binding buffer to the tube and mixing until the mixture was uniform. Then 50 ul of glass milk was added and mixed by quick inversion over a period of 10 min. The mixture was centrifuged at 7,000 g for 30 seconds, the supernatant discarded, and the pellet was washed twice with 500 ul of wash buffer. The glass milk was then washed with 500 ul of salt buffer once and centrifuged at 7,000 g for 2 min. TE (20 ul) was added to the glass pellet and the mixture incubated at 50 °C for 15 min. The glass particles were pelleted at 7,000 g for 30 seconds and the supernatant was collected. The supernatant contains the isolated DNA, which was transferred to a new tube. The glass was washed with TE (20ul) again and the supernatants (40 ul) combined.

The isolated DNA (20 ul) was used for restriction endonuclease digestion. In a total of 20 ul reaction solution 1 ul (10U) of each for Kpn I and Sca I were added, which cut the intact MT-I gene (300bp) from plasmid pMTCC (3.1 kb). The restricted DNA along with chicken MT-I and Kpn I/Sca I fragments from the plasmids were electrophoresed in a 0.8% agarose gel in TAE buffer. The gel was rinsed with 5 x SSC, denatured with 1.5 M NaCl, 0.5 NaOH, and neutralized with 1 M Tris/HCl, 1.5 M NaCl (pH 8.0) prior to transfer to nitrocellulose membrane using 10 x SSC for overnight. The blot was then baked at 80 °C for 2 hours. Prehybridization and hybridization conditions were the same as described in Maniatis *et al.* (1982). Briefly, prehybridization of the membrane was done for 3 hours at 65°C in a buffer consisting of 5 x SSC (175.3 g/L NaCl, 88.2 g/L sodium citrate, pH 7.0), 1% (w/v) SDS, 5 x Denhardt's solution, and 100 ug/ml denatured salmon sperm DNA. Then the nitrocellulose membrane was hybridized to a-^{32}P-labeled and denatured chicken MT probe DNA at 65°C

overnight. The probe was prepared by the Radprime DNA labeling system (Life Technologies, Grand Island, New York) which is designed for rapid preparation of high specific activity ^{32}P-labeled probes. Briefly, 25 ng of denatured DNA was dissolved in 20 ul of Radprime buffer in microfuge tube by heating for 5 min. in a boiling water bath, then immediately cooled on ice. One ul of each of 500 uM dATP, dTTP and dGTP was added on ice, followed by 5 ul (approximately 50 uCi) of a-^{32}P dCTP and mixed briefly. One ul (40U) of Klenow fragment was then added, and incubated for 10 min. at 37°C. The probe was boiled (denatured) for 5 min. before addition to the hybridization bottle for overnight (16 hours) hybridization at 65°C. The blot was then washed with 2 x SSC + 0.1% (w/v) SDS at 65 °C for 10 min., and then washed twice with 0.1 x SSC + 0.1% (w/v) SDS for 30 min. at 65°C. The filter was exposed to Kodak XAR-5 X-ray film overnight.

Northern Blot Analysis

In order to determine the abundance of the MT-I transcript in *C. reinhardtii*, RNA was isolated from either deflagellated (induces $\beta_2$tubulin promoter expression) or non-deflagellated wild type (CC-425) cells. For RNA isolation, 100 mililiters of cells at 10^6 cells/ml were pelleted at 3,000 x g for 5 min. and frozen in liquid nitrogen in plastic 50 ml tubes (RNase free). Then 10 mililiters of Trizol reagent (Life Technologies, Grand Island, New York) was added before the cells thawed, vortexed until homogeneous, and incubated 5 min. at room temperature. Then 2.0 mililiters of chloroform was added to the mixture, mixed for 15 seconds, and incubated 3 min. at room temperature. The mixture was transferred to Corex tube (treated with 0.1 % (v/v) DEPC water and baked overnight) and spun at 12, 000 x g for 15 min. at 4 °C.

The upper aqueous phase was transferred to a new Corex tube and 5 milliliters of isopropanol was added and incubated 10 min. at room temperature. The RNA was pelleted at 12,000 x g for 10 min. at 4 °C. The supernatant was removed, the pellet was resuspended in 10 milliliters of 75% ethanol (using DEPC-treated water to make 75% (v/v) ethanol) and pelleted at 7,500 x g for 5 min. at 4°C. RNA was vacuum dried and dissolved in RNase free water at 55 °C and stored at -80 °C.

The detachment of flagella from *C. reinhardtii* was done by the pH shock method described in the *Chlamydomonas* Sourcebook (Harris, 1988). The cells were grown in TAP medium and harvested at 5,000 x g for 5 min., then resuspended in 10 mM Tris-HCl buffer, pH, 7.8 at 25 °C and concentrated by centrifugation at 1,100 x g for 6-8 min. at 25 °C in 250 milliliters polycarbonate centrifuge tubes. The cells were immediately rinsed again by resuspension in 100 milliliters of 10 mM Tris buffer at 4 °C. All the subsequent steps were at 4°C. The suspended cells in Tris buffer were vigorously stirred with a magnetic stirrer while the pH was rapidly lowered to 4.5 with 0.5 N acetic acid. After 60 seconds, 90-100% of the cells were deflagellated (Harris, 1988). The pH of the suspension was then raised to 7.8 by additional of 0.5 N KOH. We waited one hour before RNA was isolated from deflagellated cells to allow for expression from the β_2 tubulin promoter (Davies et al., 1992).

For northern blot analysis, total RNA (15 ug) was dried under vacuum and resuspended in 50% (v/v) formamide, 2.2 M formaldehyde, 1x MOPs buffer (20 x MOPS = 0.4 M 3-[N-morpholino] propanesulfonic acid (MOPS), pH 7.0, 100 mM sodium acetate and 1 mM EDTA). The samples were heated at 55 °C for 15 min. and loaded onto a 1.0% agarose gel containing 1 x MOPS buffer, and 2.2 M formaldehyde. After the samples were separated electrophoretically

in the formaldehyde gel, the gel was rinsed with DEPC-treated water and the samples were blotted onto nitrocellulose membrane using 10 x SSC for 16 hours. The filter was then rinsed with 5 x SSC to release any bound agarose and allowed to air dry. The RNA was bound to the membrane by oven drying at 80 °C for 2 hours. Messenger RNA were detected on northern blots by hybridization to a radioactively labeled DNA fragment from the chicken MT-I gene. The probe was prepared by the Radprime DNA labeling system (Life Technologies, Grand Island, New York) as described above. The northern blot was prehybridized in 5 x SSPE (20 x SSPE = 174 g/L NaCl, 27.6 g/L $Na_2PO_4 \cdot H_2O$, 7.4 g/L EDTA; pH 7.4), 50% (v/v) formamide, 1%(w/v) SDS, and 100 ug/L sheared calf thymus DNA for three hours at 42°C. Hybridization with chicken MT-I was carried out at 42°C overnight. The blot was then washed with 2 x SSPE + 0.1% (w/v) SDS at room temperature for 5 min., and then washed twice with 0.1 x SSPE + 0.1% (w/v) SDS for 45 min. at 55°C. The hybridization filter was exposed to Kodak XAR-5 X-ray film or to a Phosphorimager screen and the intensity of bands were determined by the Phosphorimager scanner (Molecular Dynamics, Sunnyvale, CA).

Results and Discussion

Cell Growth in Response to Heavy Metals

Cell Growth in Response to Cadmium

The initial objective was to determine the effects of various Cd concentrations (in TAP medium) on the growth of *Chlamydomonas reinhardtii* Strain CC- 2137. It has been well documented that the chemical activity of Cd can vary as a function of pH and phosphate

concentration (Schulze *et al.*, 1978). A reduction (30% at 50 uM Cd) in cell growth was observed only at Cd concentrations ≥ 50 uM. Significantly, these cadmium concentrations (≥ 50 uM) also caused a visible precipitate to form in TAP medium. The effect of different concentrations of Cd on the growth of in *Chlamydomonas* liquid culture was examined to define metal concentrations that *Chlamydomonas* cells (CC-2137) can tolerate. At Cd concentration of 50 uM or less, there was no reduction in cell growth, indicating that *Chlamydomonas* (CC-2137) is tolerant to elevated Cd concentrations (Fig. 4). In this study chlorophyll accumulation was used as a measure of cell growth. Because the availability of free Cd, rather than complexed Cd , could determine its relative toxicity, the chemical activity of Cd in TAP medium was calculated using the geochemical Speciation Program SoliChem (Sposito *et al.*, 1988). As shown in Figure 5, the negative log of the chemical activity of Cd^{2+} (pCd), at total Cd concentrations ranging from 1 to 50 uM, ranged between 9.2 and 6.3. It was apparent, however, that there was a linear relationship between [Cd] and chemical activity. As previously demonstrated by Howe and Merchant (1992), phytochelatin synthesis is induced in *Chlamydomonas* stationary-phase cultures following addition of Cd at concentrations of at least 25 uM. This concentration corresponds to a chemical activity (pCd) of 7.0. Significantly, there was no obvious breaks in the chemical activity profile.

Cell Growth in Response to Buthionine Sulfoximine (BSO)

The direct involvement of phytochelatins in determining bound Cd content remains to be demonstrated. To address this issue cells were grown in the presence of Cd and BSO (buthionine sulfoximine), an inhibitor of glutathione and phytochelatin synthesis. Howe and Merchant (1992) had previously demonstrated that addition of 10 mM BSO to TAP medium containing 400 uM Cd prevented cell growth on agar plates. In these experiments, however, 400 uM Cd causes formation of precipitates in TAP medium. Furthermore, an inhibition of cell growth in liquid TAP containing up to 200 uM BSO was not observed. To determine whether or not phytochelatins protected cells from the potentially toxic affects of Cd and to avoid potential secondary effects, cell growth in the presence of reduced concentrations of Cd and BSO was monitored. As shown in Figure 6, BSO concentrations ranging from 5 to 100 uM, in the presence or absence of 40 uM Cd, had little effect on cell growth (as indicated by an increase in chlorophyll concentration).

The Effect of Buthionine Sulfoximine on Cadmium-Binding Fractions of *Chlamydomonas* Cells

When the cells were grown in the presence of 40 uM Cd plus 100 uM BSO there was a 3-fold increase in bound Cd relative to cells grown in the absence of BSO (0.334 ppm Cd, plus BSO; versus 0.111 ppm, minus BSO). Although it has not been demonstrated that the BSO concentrations used inhibit glutathione synthesis (in liquid culture), it is apparent that the BSO treatment enhanced bound Cd content, possibly by some other Cd binding fraction. To identify the cellular fraction that accounted for the increased Cd binding during treatment with BSO,

cells were grown in the presence of 40 uM Cd plus various concentrations of BSO and fractionated the cells into a soluble fraction and membrane plus cell wall fraction (100,000 x g). It was assumed that the low molecular weight and water soluble phytochelatins would only appear in the soluble fraction. The cadmium content of each fraction was measured by atomic absorption spectroscopy. As shown in Table 3, cells grown in the presence of 40 uM Cd bound approximately 13% of the total Cd available in the medium. Growth in the presence of BSO (5-100 uM) enhanced the Cd binding of cells by 2-fold. Interestingly, treatment with 5 uM BSO increased the Cd present in the soluble fraction relative to cells without added BSO.

Table 3. The Effect of the Phytochelatin Synthesis Inhibitor Buthionine Sulfoximine (BSO) on Cadmium Binding Fractions of *Chlamydomonas reinhardtii* Cells Grown in 40 uM Cadmium.

Table 3.

[BSO] (uM)	Cd Content of Soluble Fraction (ug)	Cd Content of Pellet Fraction (ug)	Total Cd Bound to Cells* (ug)
0	28.5	28.7	57.2 (12.8)
5	38.8	83.0	122 (27.2)
50	19.3	81.4	101 (22.5)
200	9.9	80.5	90.4 (20.2)

* Percentage of total Cd available in medium bound to cells. Pellet is 100,000 X g fraction. Results are the average of two experiments. Replicate data values varied by no more than 10% of the total. Values are for 100 milliliters of cell culture having identical cell numbers.

Higher BSO concentrations, however, reduced the level of Cd in the soluble fraction (34% of the control level in 200 uM BSO treated cells). These results are consistent with reduced levels of phytochelatins due to BSO inhibition of their synthesis. More important, however, is the observation that the Cd present in the pellet fraction increases nearly 3-fold in cells treated with BSO relative to the control. In fact, the majority (68%) of the Cd present in the cell was localized in the pellet fraction of cells treated with 5 uM BSO. These results suggested that a cell wall or membrane-associated Cd binding fraction other than phytochelatins is induced following BSO treatment. The identity of this Cd boundary fraction was not determined.

Magnesium-Cadmium Exchange in Chlorophyll

Heavy metal-substituted chlorophylls and related porphyrins have been known *in vitro* for a long time. Zn-porphyrins are known from chlorophyll biosynthesis (Rebeiz *et al.*, 1973), as well as from porphyrin decay; Zn^{2+} may complex to the breakdown product biliverdin (Hendry *et al.*, 1980). Cu-porphyrins are known from deep-sea sediments, and it was suggested that these substances are recent degradation products of Mg-chlorophyll. In search of a mechanism which could enhance the heavy metal binding capacity of algae, heavy metal substituted chlorophyll in algae was probed. Both the Cd and Mg content of acetone (80% v/v) extracted chlorophyll (a+b) was measured in order to determine the amounts of Cd and Mg in chlorophyll. Stoichiometrically, there should be 1 Mg for each Chl. As shown in Table 4, the molar Mg/Chl ratio ranges from 0.53 to 0.86 indicating that the accuracy of our metal/pigment measurements could be off as much as 88%. When cells were grown in the presence of 40 uM Cd (Table 4) the average Cd/Chl ratio was 0.42. Similar results (0.37 Cd/Chl) were obtained

for cells grown in the absence of Cd but to which Cd was added prior to chlorophyll extraction. These results indicate that the Cd present in chlorophyll extracts could result from Cd contamination in the media or by non-specific exchange of Mg with Cd during chlorophyll extraction. Overall, it is apparent, however, that ≥ 10% of the total bound Cd (Table 3) could be accounted by Cd exchange with Mg in chlorophyll.

Table 4. Results of Mg-Cd Exchange in Chlorophyll from Cells Grown in the Presence or Absence of Cd [40 uM]. The concentrations of Cd and Mg were measured by Atomic Absorption Spectroscopy. Chlorophyll was measured from acetone extracts.

Table 4.

Sample	Total Chl umol	Total Mg umol	Total Cd nmol	Cd/chl %	*Mg/Chl %
Ctr1	2.45	1.90	-----	-----	0.78
Ctr2	2.40	1.67	-----	-----	0.70
Ctr+Cd1	2.38	1.25	14	0.59	0.53
Ctr+Cd2	2.40	1.46	4.4	0.18	0.61
40uM1	1.80	1.04	4.4	0.24	0.58
40uM2	0.73	0.63	4.4	0.60	0.86

$MW_{Cd} = 112.4$; $MW_{Mg} = 24$; $MW_{Chl} = 900$
*Calculated as one chlorophyll to one Mg, equals 100%.
Ctr: Control cells
Ctr+Cd: 40 uM Cd added before extraction of the cells
40uM: 40 uM Cd added at the beginning of cell growth
Cd values for last three samples are at lower limits of the instrument detection

Transformation of Chicken MT-I Gene into Chlamydomonas

Chicken MT-I Gene Cloning

The chicken metallothionein MT-I structural gene was excised from pMT6c7 by digesting with EcoR I and Hind III enzymes, the size of the DNA fragment is about 300 bp. The strategy for constructing the chimaeric gene containing chicken MT-I is shown in Fig. 3. The Chicken MT-I fragment was cloned into the EcoR I and Hind III sites of the pJD55 fragment (see methods), amplified using two primers corresponding to the 3' end of the β_2-tubulin promoter and the 5' end of the 3' Ars UTR. These primers also introduced new EcoR I and Hind III restriction sites. The cloned product which contained chicken MT-I gene was named pMTCC, and confirmed by enzyme restriction digestion (Fig. 7) and Southern blot analysis (Fig. 8). Briefly, cloned pMTCC was digested with EcoR I and Hind III enzymes, and the digested DNA was electrophoresed on 0.8% agarose gel. As shown in Figure 7, the 300 bp fragment of chicken MT-I was obtained. In the Southern blot analysis, pMTCC was digested with Kpn I and Sca I enzymes which cut at the 5' and 3' end respectively of the β_2-tubulin promoter and Ars terminator. The fragment generated is about 3.1 kbp. DNA was then transferred by the Southern blot procedure and hybridized with chicken MT-I DNA. The results are shown on Figure 8 and confirmed that chicken MT-I was cloned into pMTCC.

Using the glass bead method for transformation described in chapter II, pMTCC was introduced into *Chlamydomonas* strain CC-425 (cw-15, arg-2) via cotransformation with p389. The latter plasmid contains the argininosuccinate lyase gene which complements the arg-2 lesion and enables transformants of CC-425 to grow on arginine-deficient medium. After transformation, cells were selected for growth on solid TAP medium (lacking arginine). Cells

harboring an intact copy of the MT-I gene are able to grow in TAP medium and detected by observing the development of blue color.

Southern Blot Analysis

Using Southern blot analysis, the frequency of co-transformation was determined to be about 14% by screening 14 randomly chosen transformants and identifying two that express MT-I gene. Genomic DNA from CC-425 and the transformants was prepared by methods described previously using Eluquick minilysate procedure, then digested with Kpn I and Sca I enzymes, the digested fragment is about 3.1 kbp containing the MT-I gene, β_2-tubulin promoter and Ars terminator. For Southern blot analysis, Kpn I and Sca I digested DNA was fractionated on 0.8% agarose gel, transferred to Duralon-UV transfer membranes (Stratagene, La Jolla, Ca) and hybridized with the ^{32}P-labeled probe containing 25 ng of chicken MT-I fragment. The results are shown in Figure 9. From the Southern blot analysis we can see that the probe hybridized to an internal MT-I fragment DNA in the transformant. No hybridization was observed to control cells.

Northern Blot Analysis

The level of MT-I expression in the transformed and untransformed (CC- 425) cell lines was determined by northern blot analysis. The quantity of the RNA was first confirmed by minigel electrophoresis. Fifteen microgram of total RNA was separated on a 1% agarose formaldehyde gel, and transferred to Duralon-UV transfer membranes (Stratagene, La Jolla, Ca.). The blot was then hybridized with ^{32}P-labeled Chicken MT-I DNA. A band at about 550

bases was detected in the two transfomants but not in untransformed CC-425. This band corresponds in size to that predicted for the structural region of the gene and untranslated regions of the MT-I mRNA. Northern analysis shown in Figure 10, demonstrated that the MT-I gene is expressed in transformed cells. However, we did not observe higher expression in deflagellated cells was not observed. A previous northern of the RNA sample shown in lane 6 indicated that the MT-I was expressed. It is apparent that the RNA used for lane 6 had subsequently degraded.

Analysis of Cadmium Binding in Transformants

In order to determine whether expression of MT-I affected Cd binding we grew wild type (CC-425) and transformed cells in the presence of 40 uM Cd and measured total bound Cd. Cell growth (TAP plus 50 ug arg/ml) was determined by measuring total chlorophyll. The cell growth curve is shown in Figure 11. The results show that the transformed cells have the same growth curve as the wild type cells in the absence of Cd, indicating that expression of MT-I in *Chlamydomonas* is not toxic to the cells. In the presence of Cd, the growth rate of wild type cells was approximately one third that of wild type cells in the absence of Cd. The transformed strain pMTCC4 grew at a rate similar to wild type in the presence of Cd, however, strain pMTCC8 grew at rate which was 2-fold greater than wild type (Figure 12). These results indicate that strain pMTCC8 may be less susceptible to Cd toxicity than wild type.

The amount of Cd bound to transformed (pMTCC4 and pMTCC8) and wild type (CC-425) cells was also measured. Cells grown in TAP plus 50 ug arg/ml and were harvested after 5 days, the cell pellet was washed once with a solution containing 50 uM potassium phosphate,

pH 7.0 to remove any loosely bound Cd. The Cd contents of the pellet were measured using an Atomic Absorption Spectrophotometer. The results in Figure 13 indicated that, in the presence of 40 uM Cd, the transformed cells had no increased capacity to bind Cd compared to wild type (CC-425), however, pMTCC8 cells grew twice as fast as CC-425 and bound twice as much total Cd.

Table 5. Cadmium Binding in Wild Type (CC-425) and Transformed (pMTCC4 and pMTCC8) *Chlamydomonas* Cells. Values are for 1 L Cultures and Average of two measurements. Error bars represent one standard deviation.

Table 5.

Strain	Total Chl (mg)	Total Cd (ug) (% of total in medium)	Cd/Chl (ug/mg)
CC-425	1.43	426(9.5)	298
pMTCC8	2.28	672(15)	295

Values are for 1 L cultures and average of two measurements.

Conclusion

Any mechanism that would enhance the heavy metal binding capacity of algae would improve their potential application for heavy metal removal from contaminated water. Although the present inventions are not limited to their theory of operation, there are at least

two possible mechanisms to enhance the heavy metal binding capacity of *Chlamydomonas*. These are increased levels of expression of metallothioneins or other endogenous heavy metal binding factors and expression of metallothioneins with higher affinities for heavy metal. These two possible mechanisms were examined with respect to the present invention.

It was demonstrated that the growth of the unicellular algae *Chlamydomonas reinhardtii* is not affected by cadmium concentrations (≤ 50 uM) which inhibited the growth of many higher plants (Howe *et al*, 1992). Cd concentrations that induce phytochelatin synthesis have been previously shown to increase the Cd binding capacity of cells (Howe *et al.*, 1992). Cells treated with the phytochelatin synthesis inhibitor, buthionine sulfoximine, however, bound 3-fold more Cd than untreated cells, suggesting that some component other than phytochelatin may bind Cd. The Cd binding fraction induced by buthionine sulfoximine treatment which appears to be localized in the cell membrane or cell wall fraction may not be a phytochelatin. However, higher BSO concentrations, reduced the level of Cd in the soluble fraction. These results are consistent with reduced levels of phytochelatin. At this point, *Chlamydomonas reinhardtii* has demonstrated the ability to synthesize phytochelatins in response to heavy metal exposure, and its cell wall may also sequester heavy metals.

It was determined in a survey of 12 different algal species that *Chlamydomonas* has less than half the phytochelatin content (8 nmol/mg total protein) of other algae surveyed (range from 4-45 nmol/mg total protein; average is 18 nmol/mg total protein) (Gekkler, *et al.*, 1988). These results suggested that the heavy metal binding capacity may be enhanced by higher levels of metallothionein expression. In searching these and other parameters controlling heavy metal binding capacity, an avian metallothionein gene was introduced into *Chlamydomonas*. The

avian metallothioneins have higher heavy metal binding affinities than do phytochelatins. The results here show that a chicken MT-I gene has been integrated and expressed in transgenic *Chlamydomonas*. As indicated previously, the expression of the genes encoding the MT-I and MT-II are induced following exposure to heavy metals, however it is not known whether MT-I or MT-IIs are present in algae, so a chicken MT-I was expressed under the regulation of an inducible β_2-tubulin promoter. As demonstrated in Davies *et al.* (1992), transcription from the β_2 tubulin promoter is induced several fold following deflagellation of the cell. However, northern analysis shows the strongest expression in normal cells rather than deflagellated cells. It is apparent that the β_2- tubulin was not turned on when they were deflagellated, possibly due to strain (wall-less vs. walled) differences in flagellar stability.

It has been shown that the transformed (pMTCC) *Chlamydomonas* cells can tolerate Cd concentrations of 40 uM with no reduction in cell growth unlike the wild type (CC-425) cell wall-less cells. Analysis of the heavy metal content per cell of CC-425 and pMTCC8 cells grown in the presence of 40 uM Cd indicated that the transformed cells had no increased capacity to bind Cd relative to CC- 425. pMTCC8 cells, however, grow twice as fast as CC-425 and bound twice as much total Cd. These results indicate the expression of MT-I in CC-425 reduces Cd toxicity similar to the effect observed in yeast (Hamer, 1986). It is also apparent that MT-I expression does not reduce growth due to copper depletion. Since *C.reinhardtii* may replace copper containing proteins such as plastocyanin with cytochromes (C3), its sensitivity to copper depletion may be minimal. In addition, it was apparent that wall-less cells (Table 5) bind slightly more (672 ug/L) total Cd than similar cultures of walled cells (570 ug/L, Table 3). Thus the expression of MT-I may reduce Cd toxicity effects in wall-less

cells to a greater extent than in walled cells. A question remained as to why transformant pMTCC4 did not respond in a manner similar to pMTCC8 during growth in the presence of Cd. In order to determine whether there were differences in MT-I protein levels in pMTCC4 and pMTCC8, a western blot analysis was performed using an antibody directed against *Arabidopsis* MT-II. It was observed that a ≤10 kD cross-reacting band only in pMTCC8 and not in wild type and pMTCC4 (data not shown). These results account for the apparent differences in Cd sensitivity between pMTCC4 and pMTCC8.

Figures 13 is a graph showing the removal of cadmium over time from an aqueous solution through the incremental addition of algal cells, in accordance with one embodiment of the present invention. The Y axis indicates the amount of cadmium remaining in the solution after the treatment. The time (X-axis) indicates the time of the treatment. This graph demonstrates how much dried algae is typically required for removal of the cadmium.

Figure 14 is a graph showing the pH dependency of metal binding to algal cells allowing for the regeneration of cells, in accordance with one embodiment of the present invention. This graph shows that at pH 7.0 most of the cadmium is bound or removed from the medium. As the pH is lowered to pH 3.0 the cadmium is released back into the medium. Then the pH was raised again and the cadmium was removed from the medium. This demonstrates that the cadmium binding can be regenerated.

Figure 15 is a graph showing the binding of gold, uranium and cadmium by dried algal cells as a function of pH, in accordance with one embodiment of the present invention. This graph shows the binding of gold, uranium and cadmium by dried cells as a function of pH. The cadmium binding is very pH dependent unlike gold or uranium. These results indicate that the

types of binding sites for the different elements are unique. Furthermore it shows that gold and uranium binding is essentially quantitative.

Figure 16 is a graph showing a comparison of the pH dependent cadmium binding properties of dried walled (normal) cells and dried cell wall-less cells at high ionic strength, in accordance with one embodiment of the present invention. This graph compares the pH dependent cadmium binding properties of dried walled (normal) cells (cc-2137) and dried cell wall-less cells (cc-425) at high ionic strength (0.01 M). Both cell types have similar cadmium binding properties. The results are relevant to the transformant since it was generated in a wall-less strain. There has previously been no experimental work that shows this similarity between walled and wall-less cells for cadmium binding. In general it was believed that the wall bound all the cadmium. These results could not be predicted, therefore.

Summarized Results Of Laboratory Studies Designed To Elucidate Feasibility Of Using Chlamydomonas Reinhardtii Biomass As A Potential Commercial Metal Sorbent.

Part 1 - Thermal Stability Studies

Part 2 - Tea Bag Approach

Part 3 - Nylon Bag Approach

Part 4 - Metal Binding Studies Using CC-2137 Biomass

Part 5 - Metal Binding Studies Using Modified CC-2137 Biomass Part 6 - pH Cycling Of Cells (Sorption-Desorption Cycles)

Introduction

The following experiments were designed with a view to testing the algal biomass in a somewhat realistic setting and to challenge the capabilities of the biosorbent in an attempt to better estimate optimal performance. The experimental design and procedures were altered and/or abbreviated to accommodate logistical and other circumstances as necessary.

Mean concentrations of major ions in N. America according to R. Wetzel ('Limnology', 2 d ed., 1983, Saunders Publishers, Philadelphia) are:

21 ppm Ca^{2+}, 5 ppm Mg^{2+}, 9 ppm Na^{+}, 1.4 ppm K^{+}, 68 ppm HCO_3^{-},20 ppm SO_4^{2-}, 8 ppm Cl^{-}, I ppm NO_3^{-},,9 ppm SiO_2

Recipe for simulated natural water (SNW):

I 00 mg KNO_3 (molecular weight = 101.11)

* 836 mg $MgCl_2$ (molecular weight = 203.30)

* 1720 mg $CaSO_4$ (molecular weight = 172)

* 2472 mg Na_2SiO_3 (molecular weight = 284.29)

The salts were dissolved in 20 L deionized water. The pH of SNW was 9.3.

Some general observations regarding the cells: there were differences in color, texture and clumping (particle sizes) within and among batches of cells. An attempt was made to mix and grind cells to a more uniform distribution and average out the properties. However, differences between batches of cells remained and their effects were not entirely determinable in this study.

There were distinct differences between the physical properties of unmodified and crosslinked cells. The crosslinked cells possessed greater density and exhibited a darker green coloration.

No significant sorption of metals to the glass flasks, used as reaction vessels, was observed.

Part I - Thermal Stability Studies

Known weights of cells were subjected to dry heat at 100, 200 and 300 degrees Celsius for 1, 2 and 5 hrs, each.

Gradual changes in color were observed (green to brown) over time, especially at 200°C.

At 300°C, unmodified cells were charred black and fused after one hour of heating. Cells were withdrawn from the oven in this case and no treatments at that temperature were administered for the crosslinked cells.

Metal binding studies were conducted only for samples with treatment times of I hour. Mixtures were contained in 50 mililiters covered tubes containing 0.025 g dried cells and - 0. I mm Cd (i.e., cell density = 0.5 g/L). The solution pH was set to 5. The tubes were set on a shaker and agitated for 60 minutes.

The performance of the heat-treated cells subjected to is summarized below-

Sample	*% Cd removed*	*Sample*	*% Cd removed*
Unmodified 100°C	25 ± 4	Crosslinked 100°C	26 ± I
Unmodified 200°C	22 ± 3	Crosslinked 200°C	23 ± I
Unmodified 300°C	*15* ± 1	Crosslinked	26 ± 0

Unmodified 27 ± 1

Hence, it appears that very strong heat treatment resulted in some loss of metal binding performance. However, it is encouraging to note that even at 100° C little damage was done over an hour to the unmodified and crosslinked cells. At 200'C, detrimental effects of heat were evident.

It is also noteworthy that there was little difference between the performances of the unmodified and crosslinked biomass for the biosorption of Cd.

Crosslinking provided no apparent structural advantage against heat treatment.

Part 2 - Tea Bag Approach

The containment of lyophilized cells was attempted by enclosing a known weight of cells within a "tea bag". This approach was inherently flawed in that the mesh size of the tea bags was much larger than the size of the cells resulting in leakage of cells into the surrounding solution immediately upon immersion of the "tea bags". This approach was abandoned.

Part 3 - Nylon Bag Approach

Fine mesh fabrics manufactured by Small Parts Inc. (5 and 10 microns mesh) were used to prepare "bags" containing known weights of cells. Two different techniques were employed to prepare the bags:

1) 3" squares of fabrics were cut from the nylon swatches. 0.2 g of dry cells were placed in the center of the squares and the squares were rolled (as one might a cigarette) with the cells located as close to the center as possible. Each "roll" was then folded in half and the two ends tied tightly together with a self-locking plastic tie.

2) 3" squares of fabrics were cut from the nylon swatches. 0.025 g of dry cells were placed in the center of the squares and the ends of the square were brought together and tied with a self-locking plastic tie.

While using technique (1) it was found that the presence of several layers of fine mesh nylon surrounding the cells slowed the diffusion of ions across the barrier in spite of constant agitation of the flasks. Bags suspended in 250 mililiters of 0. I mM Cd solutions SNW at pH 6.5 achieved 25% removal after several days. Up to 80% removal was expected based upon previous work with free-cell suspensions. It was also likely that since a large number of cells

were compressed in the rather small space, effective surface area was low. Moreover, some cells leaked out in spite of the rather tightly bound fasteners.

Other observations: the solution in flasks with (bag+cells) turned milky white in contrast to the clear solution in the flasks holding "blank" bags (i.e., no cells). It was inferred that the color change was not a precipitation reaction, but a result of bacterial contamination.

There was no difference between the performance of the two mesh sizes and the fabric, along with the plastic fasteners, seemed to binding no Cd-

While using technique (2), 1/8 the weight of cells, 0.025 g, were enclosed in the same amount of space as in the former technique. Moreover fewer folds (1 or 2) existed allowing for freer movement of cells and solution within the bag. As a result binding was seen to occur more quickly, but the performance of the biomass still appeared to be hampered.

Since the nylon fabric appeared to trap the algal cells well, the fabric was used to simply filter the cells out of a free cell suspension. The set up used the same materials in a different arrangement. Thus, it was attempted to take full advantage of the fast reactions between the metals and cells, while economizing on the expensive nylon material.

Part 4 - Metal Binding Studies Using Unmodified CC-2137 Biomass

Reaction volume was decreased to 100 milliliters. The cell density was adjusted to approximately 0.5 g/L. Acid (trace metal grade HCl) and base (KOH) solutions were prepared to adjust pH to 5 in all solutions after mixing in free cells.

Commercial metal standard solutions (I 000 ppm) were added in the following quantities to individual flasks bringing concentrations to approximately 0.1 mM.

Cadmium (Cd)	1.1 ml	(I Io ppm)
Calcium (Ca)	0.1 ml	(4 ppm)
Copper (Cu)	0.64 ml	(6-4 ppm)
Gold (Au)	1.97 ml	(I 9.7 ppm)
Uranium (U)	2.4 ml	(24 ppm)
Lead (Pb)	20.7 ml	(20.7 ppm)
Nickel (Ni)	0.59 ml	(5.9 ppm)
Zinc (Zn)	0.65 ml	(6.5 ppm)

Two mililiter aliquots were taken from solution at 30 min and 2 hours and 2 days following the start of the experiment. The aliquots were diluted with acidified SNW. Samples were analyzed using Inductively Couple Plasma (ICP) optical emission spectroscopy. Calibration standards were prepared in the same solution matrix as samples to prevent errors that arise from such differences.

Antimicrobial agent PPM™ was used (0. 1 % v/v) to prevent bacterial and fungal contamination of the mixtures over 2 days. The effectiveness of the compound was sustained for over I week following the start of the experiment. The presence of the compound did not appear to affect the quantity of metals bound.

Flasks were set on orbital shakers to provide maximum possible constant agitation to the suspensions (150 rpm).

There were significant differences in the physical properties, especially clumping, among different flasks.

Color changes were observed in the flasks containing copper and gold: The settled cells in the flask exhibited a darker green probably due to the presence of the metal on the surface of the cells. The solutions containing gold all showed strong purple coloration. The reason for this phenomenon is still under investigation. It is not yet clear whether the presence of PPM™ was directly responsible for the formation of a colored solution complex with the residual gold in solution and whether this affects the biosorption of gold.

No significant changes in pH were recorded over 2 days in most of the mixtures. A drop of I pH unit in flasks containing gold was noted. This change is not likely to influence the binding of gold. Upward changes up to 0.5 units were measured in some solutions containing uranium and zinc as the sole target metal. No adverse effect on the metal binding was apparent.

Comparative And Competitive Metal Binding Studies

Figures 17 - 26 show the results of several metal binding studies using compositions and methods of the present invention.

Figure 17 is a graph showing the removal of Cd from different solutions containing competing metal ions at pH 5. Figure 17 shows a reduction in Cd sorption was measured due to all metals tested, to varying levels. The strongest competitors to Cd binding were Au, U, Pb and Cu in that order. On the other hand, Ni, Zn and, contrary to previous experience, Ca, appear to reduce Cd binding by similar extents. Performance of the algae exceeds prediction (from earlier work) by 35%, on average. The ICP gave good RSD values (< 5%, indicating good precision in measurement) and low standard deviation between replicates, except for some multimetal mixture samples.

Figure 18 is a graph showing the removal of metals from multimetal ion mixtures at pH 5. Figure 18 shows that the order of affinity is Au > U > Pb > Cu > Zn > Ni > Cd. This correlates well with results reported in the literature, except for the lower affinity for Cd. In many studies the biosorption of Cd was greater than or comparable to that of Zn and Ni. In addition, Ca was present at a much higher concentration than other elements in all mixtures and was not well measured by ICP due to poor calibration and / or high background. Measurements using Flaine Atomic Absorption Spectroscopy revealed no significant changes in the concentration of Ca from the starting value. Approximately 0.4 mmol metal sorbed (total) per gram of dry cells from Multimetal mixtures. Sorption was completed within 2 hours for Cd, Ni & Zn and even some desorption occurred over 2 days. For Cu, U, Pb and Au, sorption was completed between 2 hours and 2 days.

Figure 19 is a graph showing the removal of metals from two-metal ion mixtures (Cd and another metal) at pH 5. Figure 19 shows that similarities in biosorption processes of Cd, Ni, and Zn were indicated in the removal of these metals from (Cd+M) mixtures. No removal

of Ca was measured; the solution concentration did not change significantly from the initial value. It should be noted again that all solutions contained relatively high concentrations of Ca. Hence, all (Cd+M) mixtures were, in fact, three-metal mixtures with Ca as the third ion.

Figure 20 is a graph showing the removal of uranium from different metal ion mixtures at pH 5. Figure 20 shows that uranium clearly out-competes Cd; with no loss of U sorption in the presence of Cd. Performance trails prediction (from earlier work) by 10%, on average. 100% removal of U was expected in all cases. Although not limited to the theory of the invention, there was probably no significant competition from other ions to uranium biosorption, resulting in this diminished performance of the biomass.

Figure 21 is a graph showing the removal of gold from different metal ion mixtures at pH 4.3. Figure 21 shows that a somewhat higher sorption of Au occurred in presence of Cd than from solutions containing only Au. Although not limited to the theory of the invention, it is believed gold binding probably suffered little or no significant competition from other ions. The solution pH dropped by one unit from initial value over 2 days. This change is not likely to influence the binding of gold.

Figure 22 is a graph showing the removal of Cd from different solutions containing competing metal ions at pH 5. Figure 22 shows that ICP analysis generated very good RSD values (<2%). Ca was present at a higher concentration than other elements in all mixtures. Ca was not well measured by ICP due to poor calibration and/or high background.

Figure 23 is a graph showing the removal of metals from multimetal ion mixtures at pH 5.

Figures Relating To Metal Binding

Figure 17 is a graph showing a comparison of several competing ions in the removal of cadmium in accordance with one embodiment of the present invention. Figure 17 shows that a reduction in Cd sorption was measured due to all metals tested, to varying levels. The strongest competitors to Cd binding were found to be Au, U, Pb and Cu in that order. In contrast, Ni, Zn and, contrary to previous experience, Ca were found to reduce Cd binding by similar extents. The performance to algae exceeds prediction (from thesis work) by 35%, on average. The ICP showed good RSD values (< 5%, indicating good precision in measurement) and low standard deviation between replicates, except for some Multimetal mixture samples.

Figure 18 is a graph showing a comparison of the removal of metals from a multimetal mixture in accordance with one embodiment of the present invention. Figure 18 shows the order of affinity to be: Au > U > Pb > Cu > Zn > Ni > Cd. This correlates well with results reported in the literature, except for the lower affinity for Cd. In many studies, the biosorption of Cd was greater than or comparable to that of Zn and Ni. Ca was present at a much higher concentration than other elements in all mixtures and was not well measured by ICP due to poor calibration and / or high background. Measurements using Flame Atomic Absorption Spectroscopy revealed no significant changes in the concentration of Ca from the starting value. It was found that approximately 0.4 mmol metal sorbed (total) per gram of dry cells from Multimetal mixtures. The sorption was completed within 2 hour for Cd, Ni & Zn and even some desorption occurred over 2 days. For Cu, U, Pb and Au, sorption was completed between 2 hours and 2 days.

Figure 19 is a graph showing a comparison of the removal of metals from a multimetal mixture in accordance with one embodiment of the present invention. Figure 19 shows that similarities in the biosorption processes of Cd, Ni, and Zn were indicated in the removals of these metals from (Cd+M) mixtures. No removal of Ca was measured and the solution concentration did not change significantly from the initial value. It should be noted, again that all solutions contained relatively high concentrations of Ca. Hence, all (Cd+M) mixtures were, in fact, three-metal mixtures with Ca as the third ion.

Figure 20 is a graph showing the removal of uranium from different metal mixtures in accordance with one embodiment of the present invention. Figure 20 shows that uranium clearly out-competes Cd with no loss of U sorption in the presence of Cd. Performance trails prediction (from thesis work) by 10% on average. One hundred percent (100%) removal of U was expected in all cases. There was probably no significant competition from other ions to uranium biosorption, resulting in this diminished performance of the biomass.

Figure 21 is a graph showing the removal of gold in accordance with one embodiment of the present invention. Figure 21 shows that a somewhat higher sorption of Au occurred in the presence of Cd than from solutions containing only Au. It is believed that gold binding probably suffered little or no significant competition from other ions. The solution pH dropped by one unit from the initial value over 2 days. This change is not likely to influence the binding of gold.

Figure 22 is a graph showing the removal of cadmium against competing ions, in accordance with one embodiment of the present invention. Figuree 22 shows that ICP analysis generated very good RSD values (<2%). Ca was present at a higher concentration than other

elements in all mixtures. Ca was not well measured by ICP due to poor calibration and/or high background.

Figure 23 is a graph showing removal of metals from a mixture of metals, in accordance with one embodiment of the present invention.

Figure 24 is a graph showing the removal of metals from two-metal mixtures, in accordance with one embodiment of the present invention. Figure 24 shows once again, that the removal of most metals was about half that from mixtures of unmodified cells. Hence, there appeared to be no effect of crosslinking on the biosorption performance of cells. It is interesting to note that even though the cell density was halved from 0.5 g/L (for the unmodified cells) to 0.25 g/L for the crosslinked cells, there was no consequent reduction in the percentage of Au sorbed. A 20% to 25 % reduction in the sorption of U was measured in the presence of competing ions. This result clearly demonstrates the lower affinity of the biomass for uranium, compared to gold. In comparison to non-linked cells, crosslinking appeared to reduce the rate of sorption; "equilibrium" values were attained between 2 hrs and 2 days for all metals in all samples. However, this phenomenon could also be attributed to the lower cell density resulting in a smaller number of available binding sites.

Figure 25 is a graph showing the removal of uranium from different mixtures, in accordance with one embodiment of the invention. Figure 25 shows that there was some loss of U sorption when measured in the presence of Cd and other ions.

Figure 26 is a graph showing the removal of gold from different mixtures, in accordance with one embodiment of the invention. Figure 26 shows that the sorption of gold was

unaffected by presence of other ions in two/multi-metal solutions and by the change in cell density. The solution pH dropped by one unit from the initial value over two days.

SUMMARY OF RESULTS:

1. We observed reduced cadmium binding at pH 5.0 relative to neutral pH conditions as predicted by our previous work. In contrast, the binding of gold (Au) and uranium (U) was nearly quantitative at pH 5.0, even in the presence of potentially competing metal ions. For each gram of dried cells, about between 0.05 and 0.1 grams of uranium was bound.

2. We also determined whether accelerated aging of the dried cells would effect their metal binding properties. The accelerated aging studies indicated that the dried algae were quite stable. One hour heat treatments at 100 or 200 °C had little effect on cadmium binding. Cross-linking of cells with glutaraldehyde, however, reduced cadmium binding by 50%. Significantly, the cross-linking had little effect on gold or uranium binding. In fact, the cross-linked material efficiently bound only uranium and gold. This increased metal binding selectivity may be advantageous for the mining industry.

In view of the foregoing disclosure, it will be within the ability of one skilled in the field of the invention to make modifications to the present invention, such as through the substitution of equivalent compositions and method steps, so as to be able to practice the invention within the scope reflected in the appended claims.

What is claimed is:

1. A transgenic algal cell, said transgenic algal cell comprising:

a transgenic algal cell of the genus Chlamydomonas and comprising reproductive genetic material comprising a nucleotide sequence, said nucleotide sequence capable of expressing chicken Type I Metallothionein.

2. A transgenic plant cell according to claim 2 wherein said transgenic algal cell is from the strain Chlamydomonas reinhardtii.

3. A transgenic plant cell according to claim 2 wherein said transgenic algal cells are selected from the strains of Chlamydomonas viable in marine environments and the strains of Chlamydomonas viable in frozen environments.

4. A transgenic plant cell according to claim 2 wherein said transgenic algal cells are in a dried state.

5. A method of removing metal from an aqueous medium containing at least one dissolved or suspended metals, said method comprising the steps:

(1) bringing into contact with said aqueous medium transgenic algal cells of the genus Chlamydomonas comprising reproductive genetic material comprising a nucleotide sequence, said nucleotide sequence capable of expressing the metal-binding protein chicken Type I Metallothionein in said transgenic algal cells;

(2) maintaining said transgenic algal cells in said aqueous medium so as to allow said at least one metal to bind to said metal-binding protein, so as to produce a metal-bound adduct of said metal-binding protein; and

(3) removing said transgenic algal cells from said aqueous medium.

6. A method according to claim 6 wherein said transgenic algal cells are from the strain Chlamydomonas reinhardtii.

7. A method according to claim 6 wherein said transgenic algal cells are selected from the strains of Chlamydomonas viable in marine environments and the strains of Chlamydomonas viable in frozen environments.

8. A method according to claim 6 wherein said transgenic algal cells are in the form of living cells viable in said aqueous medium.

9. A method according to claim 6 wherein said transgenic algal cells are in a dried state prior to introduction into said aqueous medium.

10. A method according to claim 6 wherein said at least one metal is selected from the group consisting of cadmium, copper, zinc, gold and uranium.

11. A method according to claim 6 wherein said aqueous medium in step 3 has an initial pH; additionally comprising the step:

(4) altering said pH of said transgenic algal cells removed from said aqueous medium in step 3 to a lower pH so as to cause said at least one metal to be released from said metal-binding protein, so as to regenerate said metal- binding protein.

12. A method according to claim 11 wherein said initial pH value is about 7 and said lower pH value is about 2.

13. A method according to claim 11 additionally comprising repeating steps 1 through 3 with said metal-binding protein regenerated in step 4.

14. A method according to claim 6 wherein said aqueous medium is below ground and wherein said transgenic algal cells are immobilized below ground during steps 1 and 2, and brought above ground prior to carrying out step 3.

15. A method according to claim 11 wherein said aqueous medium is below ground and wherein said transgenic algal cells are immobilized below ground during steps 1 and 2, and brought above ground prior to carrying out steps 3 and 4.

16. A method of separating a first metal selected from the group consisting of gold uranium, and mixtures thereof, from at least one second metal, in an aqueous medium, said method comprising the steps:

(1) bringing into contact with said aqueous medium transgenic algal cells of the genus Chlamydomonas comprising reproductive genetic material comprising a nucleotide sequence, said nucleotide sequence capable of expressing the metal-binding protein chicken Type I Metallothionein in said transgenic algal cells;

(2) maintaining said transgenic algal cells in said aqueous medium at an initial pH so as to allow said first metal and said at least one second metal to bind to said metal-binding protein, so as to produce a metal-bound adduct of said metal-binding protein comprising both said first metal and said at least one second metal;

(3) altering said initial pH of said transgenic algal cells to a lower pH so as to cause said at least one second metal to be released into a solution from said metal-bound adduct of metal-binding protein; and

(4) separating said transgenic algal cells from said solution containing said at least one second metal.

17. A method according to claim 16 wherein said transgenic algal cells are from the strain Chlamydomonas reinhardtii.

18. A method according to claim 16 wherein said transgenic algal cells are selected from the strains of Chlamydomonas viable in marine environments and the strains of Chlamydomonas viable in frozen environments.

19. A method according to claim 16 wherein said transgenic algal cells are in the form of living cells viable in said aqueous medium.

20. A method according to claim 16 wherein said transgenic algal cells are in a dried state prior to introduction into said aqueous medium.

21. A method according to claim 16 wherein said at least one second metal is selected from the group consisting of cadmium, copper and zinc.

22. A method according to claim 16 wherein said initial pH is about 7.

23. A method according to claim 16 additionally comprising the step:

(5) further lowering said pH of said transgenic algal cells removed from said aqueous medium in step 4 to a still lower pH so as to cause said first metal to be released from said metal-binding protein, so as to regenerate said metal-binding protein.

24. A method according to claim 23 wherein said pH to which said transgenic algal cells are lowered in step 5 is below 3.

25. A method according to claim 16 additionally comprising repeating steps 1 through 3 with said metal-binding protein regenerated in step 5.

26. A method according to claim 16 wherein said aqueous medium is below ground and wherein said transgenic algal cells are immobilized below ground during steps 1 and 2, and brought above ground prior to carrying out step 3.

27 A method according to claim 23 wherein said aqueous medium is below ground and wherein said transgenic algal cells are immobilized below ground during steps 1 and 2, and brought above ground prior to carrying out steps 3 and 4.

28. A method for producing transformed algal cells that contain and express a class II metallothionein gene comprising the steps of:

(1) excising an MT-I gene from a plasmid containing an MT-I gene;

(2) generating cloning sites on a plasmid encoding a promoter and a translation termination site;

(3) amplifying the DNA in the region between said promoter gene and said termination site;

(4) cloning the MT-I gene into the plasmid containing said amplified DNA to produce a recombinant plasmid;

(5) ligating the MT-I gene into the recombinant plasmid at the cloning sites; and

(6) transforming said algal cells with the recombinant plasmid containing the MT-I gene.

29. The method of claim 1 wherein the algal cells are of the genus *Chlamydomonas.*

30. A transgenic algae produced by the method of claim 1.

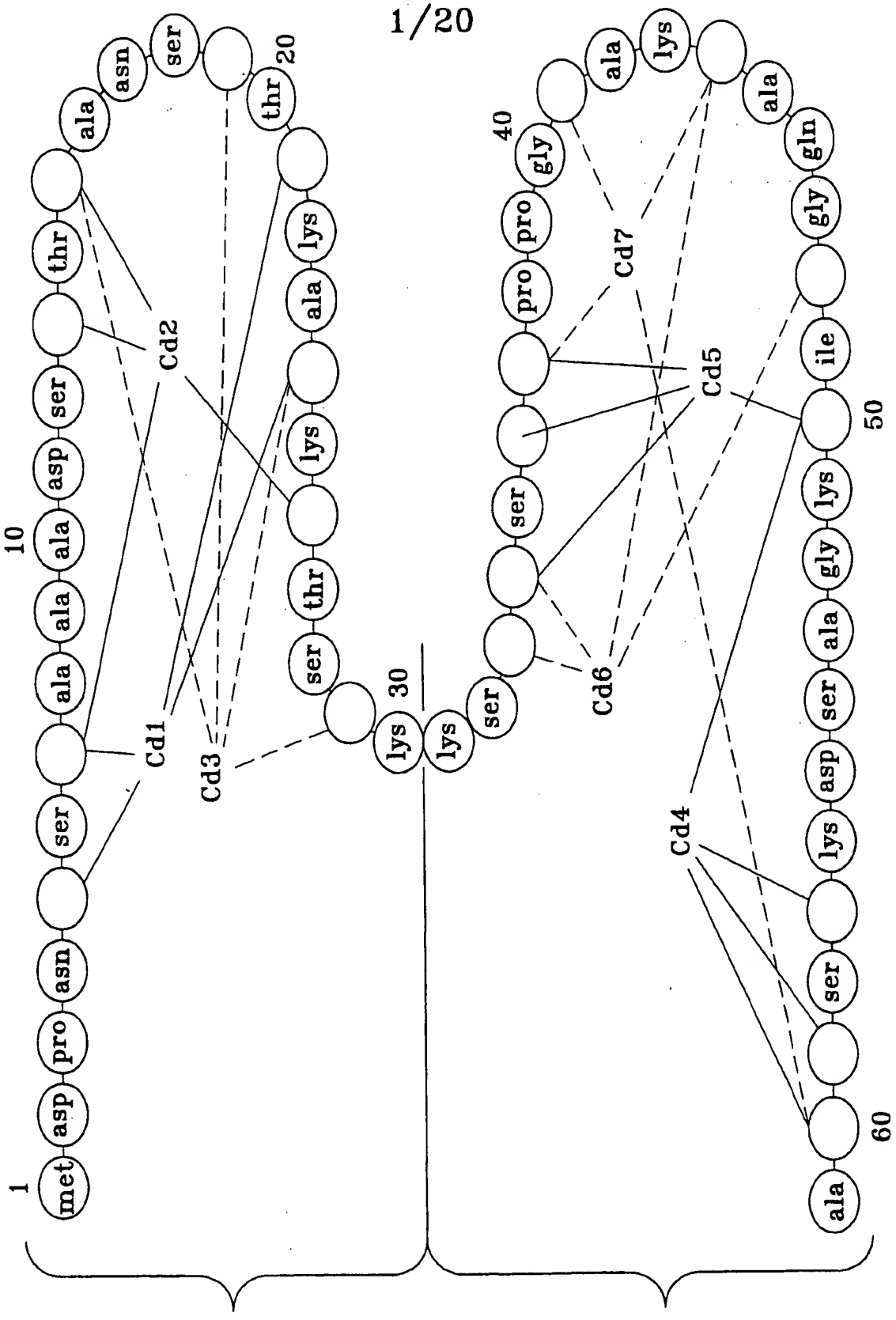

met
asp
pro
asn
ser
ala
ala
ala
asp
ser
thr
ala
asn
ser
thr
lys
ala
lys
thr
ser
lys
lys
ser
pro
pro
gly
ala
lys
ala
gln
gly
ile
lys
gly
ala
ser
asp
lys
ser
ala
Cd1
Cd2
Cd3
Cd4
Cd5
Cd6
Cd7
1
10
20
30
40
50
60

FIG-1B FIG-1A

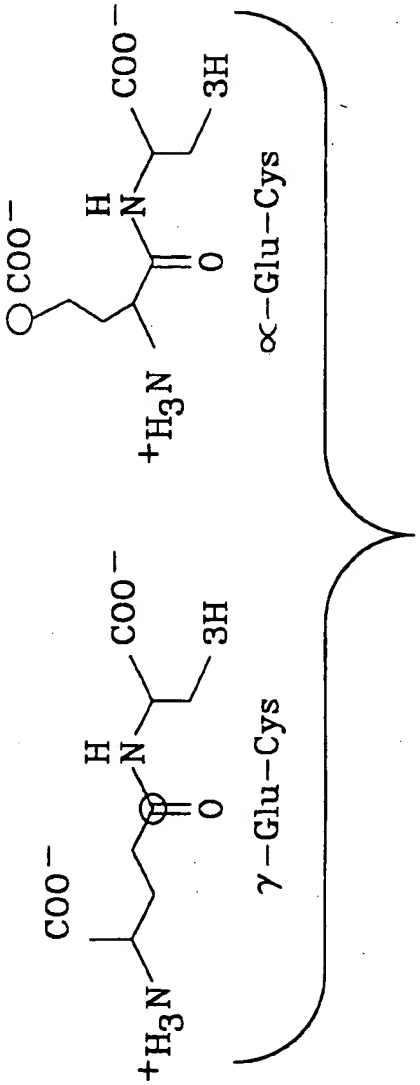

COO⁻
H
N
O
COO⁻
3H
+H3N
γ-Glu-Cys
∝-Glu-Cys

FIG-2A

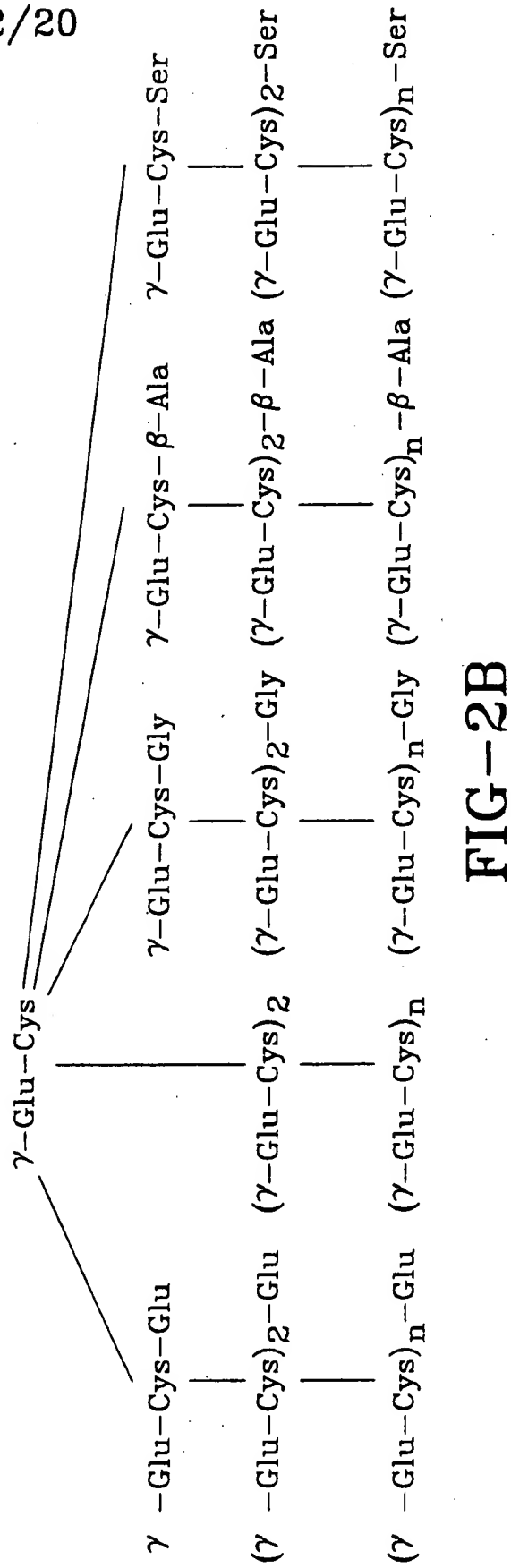

γ-Glu-Cys
γ-Glu-Cys-Gly
γ-Glu-Cys-β-Ala
γ-Glu-Cys-Ser
γ-Glu-Cys-Glu
(γ-Glu-Cys)2-Gly
(γ-Glu-Cys)2-β-Ala
(γ-Glu-Cys)2-Ser
(γ-Glu-Cys)2
(γ-Glu-Cys)n-Gly
(γ-Glu-Cys)n-β-Ala
(γ-Glu-Cys)n-Ser
(γ-Glu-Cys)2-Glu
(γ-Glu-Cys)n-Glu
(γ-Glu-Cys)n

FIG-2B

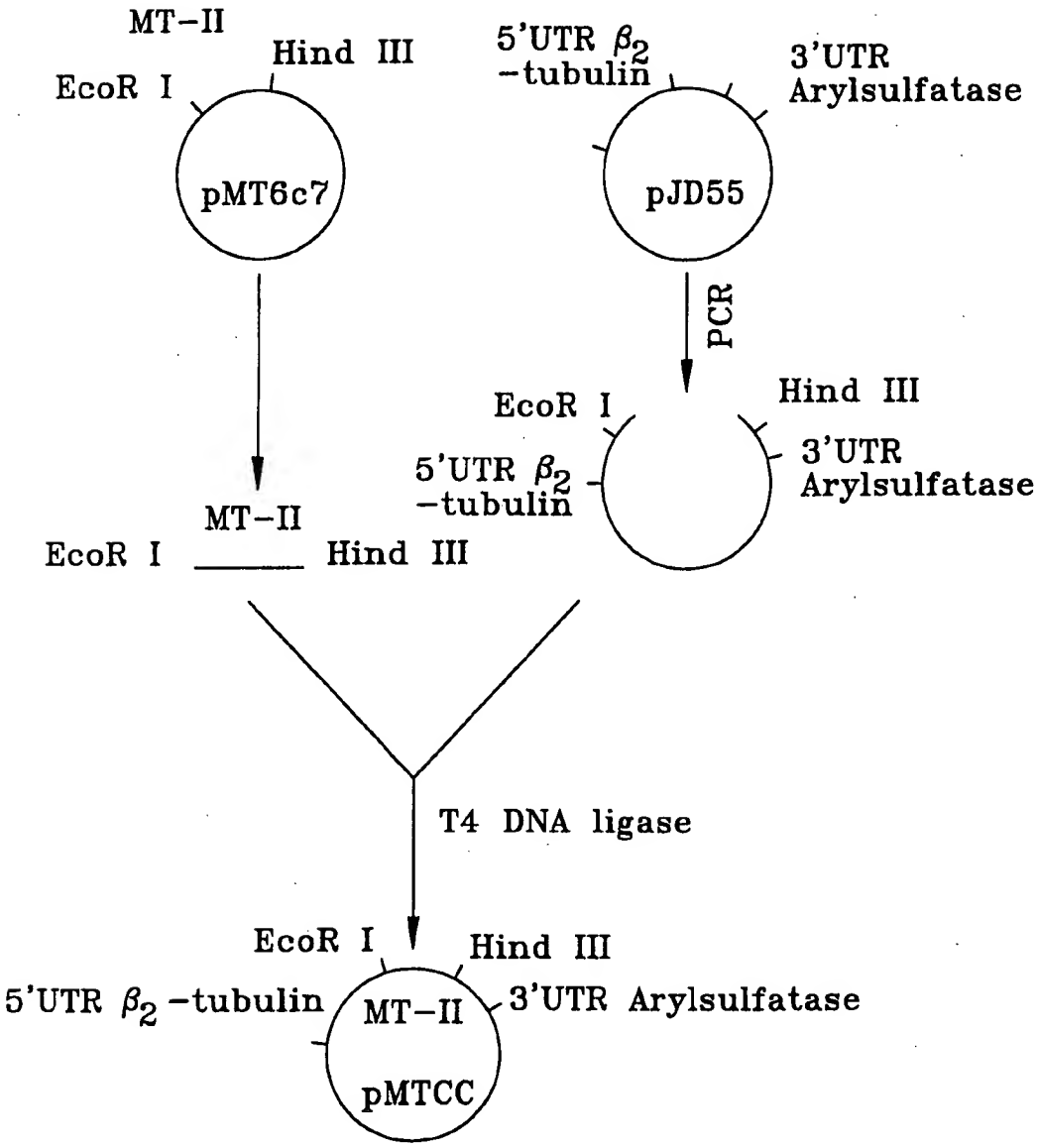

MT-II
Hind III
EcoR I
pMT6c7
5'UTR β_2 -tubulin
3'UTR Arylsulfatase
pJD55
PCR
EcoR I
Hind III
5'UTR β_2 -tubulin
3'UTR Arylsulfatase
MT-II
EcoR I
Hind III
T4 DNA ligase
EcoR I
Hind III
5'UTR β_2 -tubulin
MT-II
3'UTR Arylsulfatase
pMTCC

FIG-3

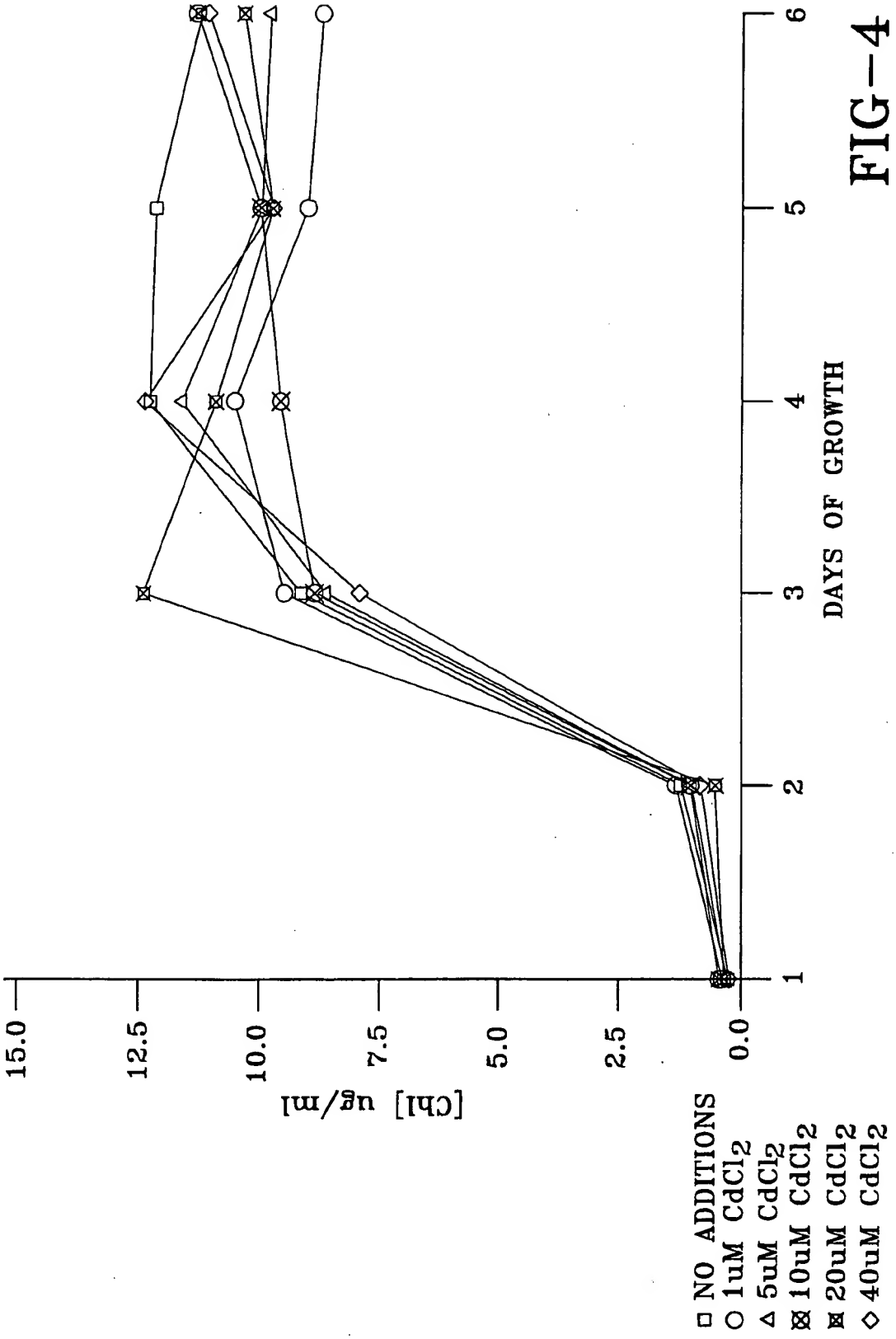
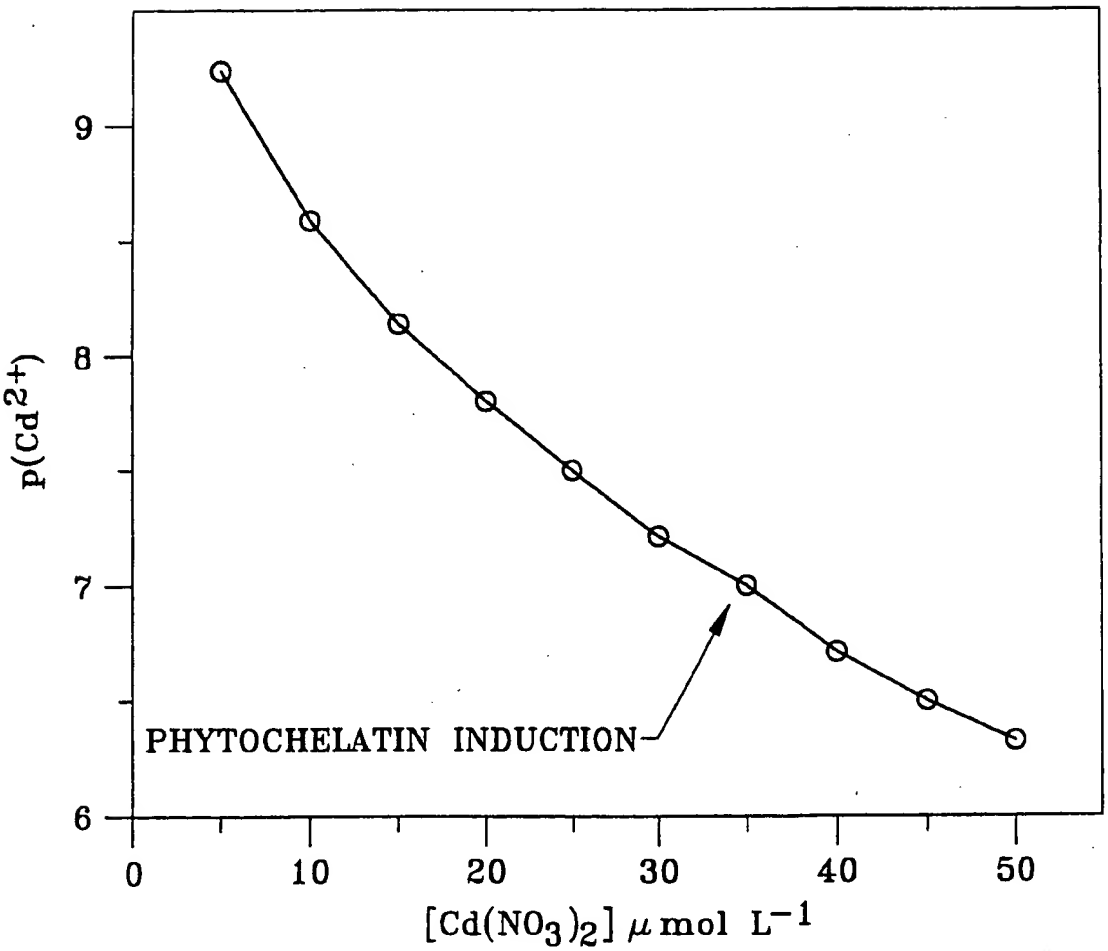

15.0
12.5
10.0
7.5
5.0
2.5
0.0
[chl] ug/ml
1
2
3
4
5
6
DAYS OF GROWTH
□ NO ADDITIONS
○ 1uM CdCl2
△ 5uM CdCl2
⊠ 10uM CdCl2
▣ 20uM CdCl2
◇ 40uM CdCl2

FIG-4


9
8
7
6
p(Cd2+)
PHYTOCHELATIN INDUCTION
0
10
20
30
40
50
[Cd(NO3)2] μmol L-1

FIG–5

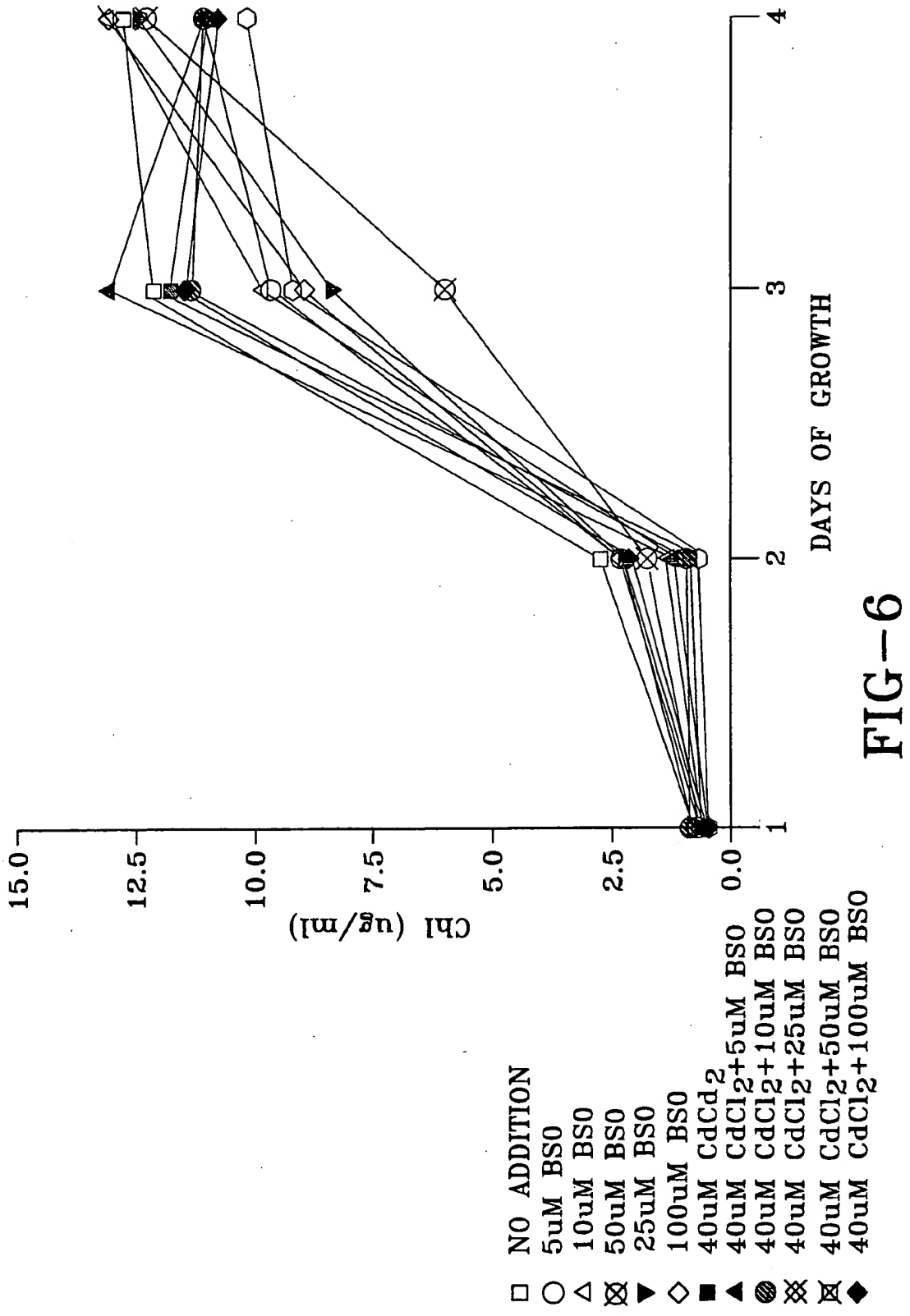

Chl (ug/ml)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
1
2
3
4
DAYS OF GROWTH
NO ADDITION
5uM BSO
10uM BSO
50uM BSO
25uM BSO
100uM BSO
40uM CdCd2
40uM CdCl2+5uM BSO
40uM CdCl2+10uM BSO
40uM CdCl2+25uM BSO
40uM CdCl2+50uM BSO
40uM CdCl2+100uM BSO

FIG-6

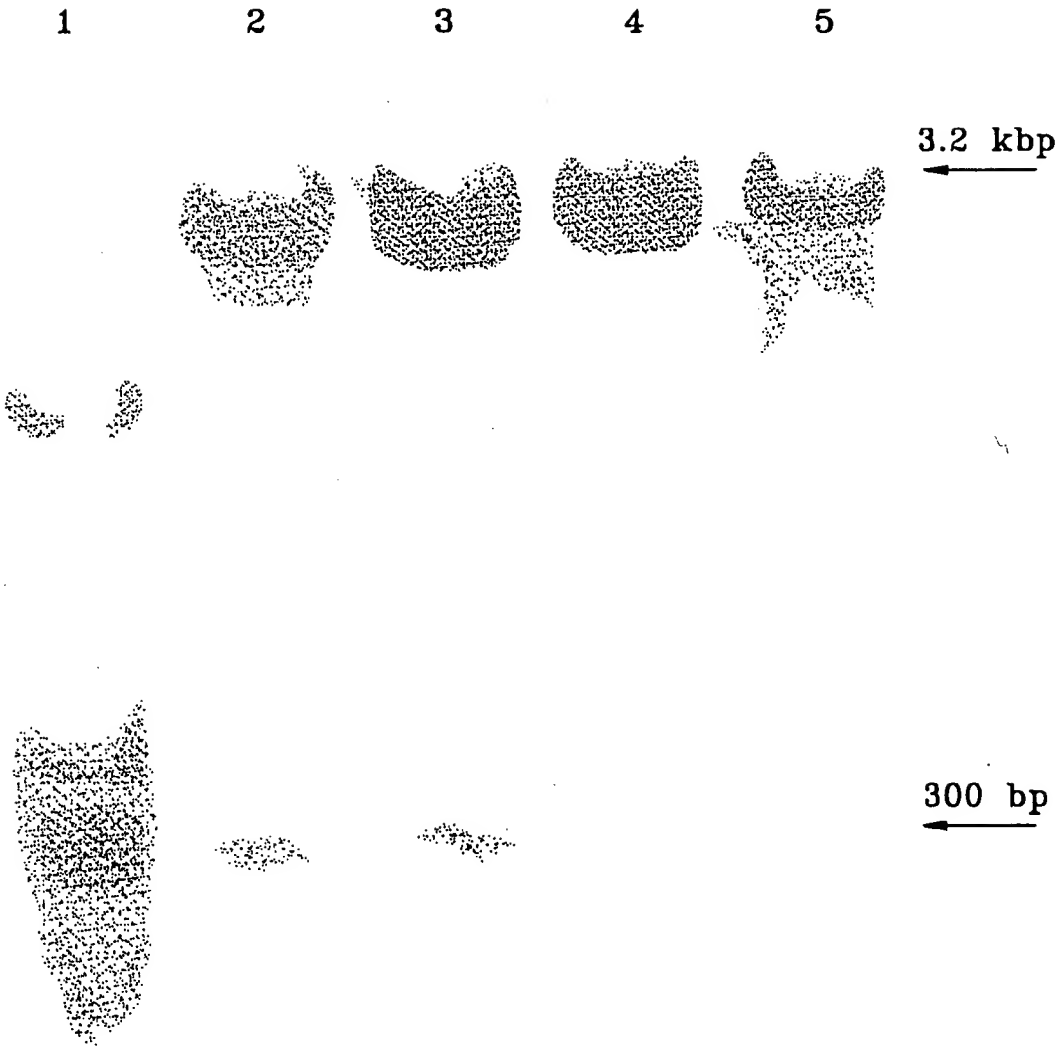


FIG-7

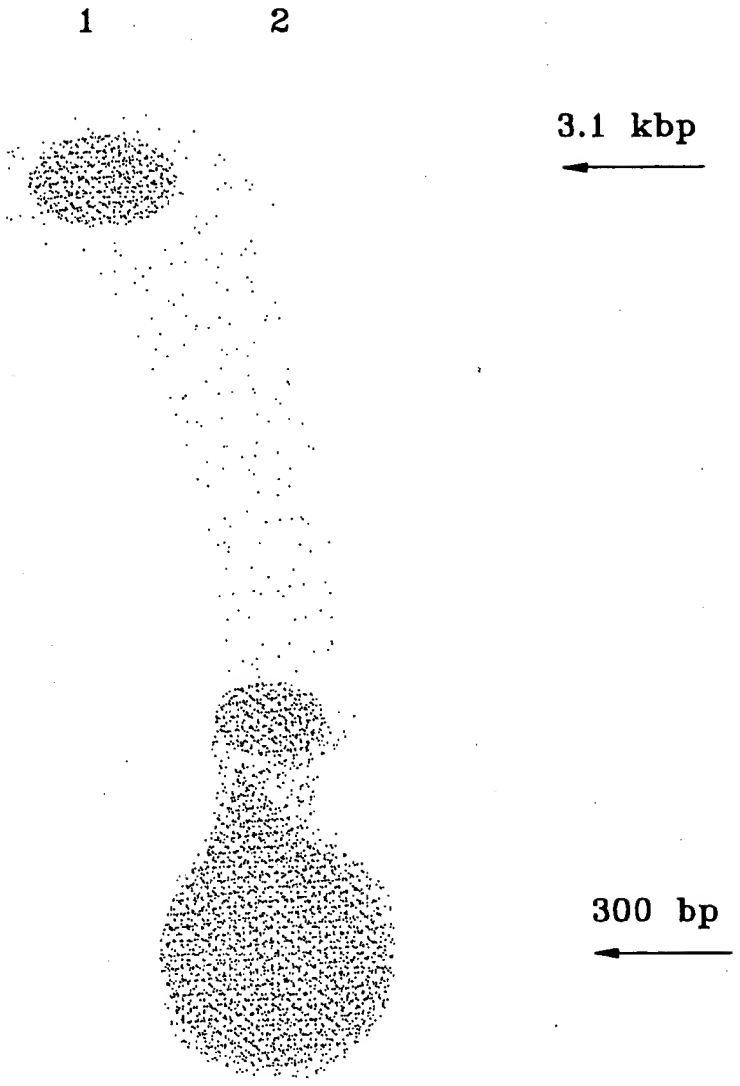


FIG-8

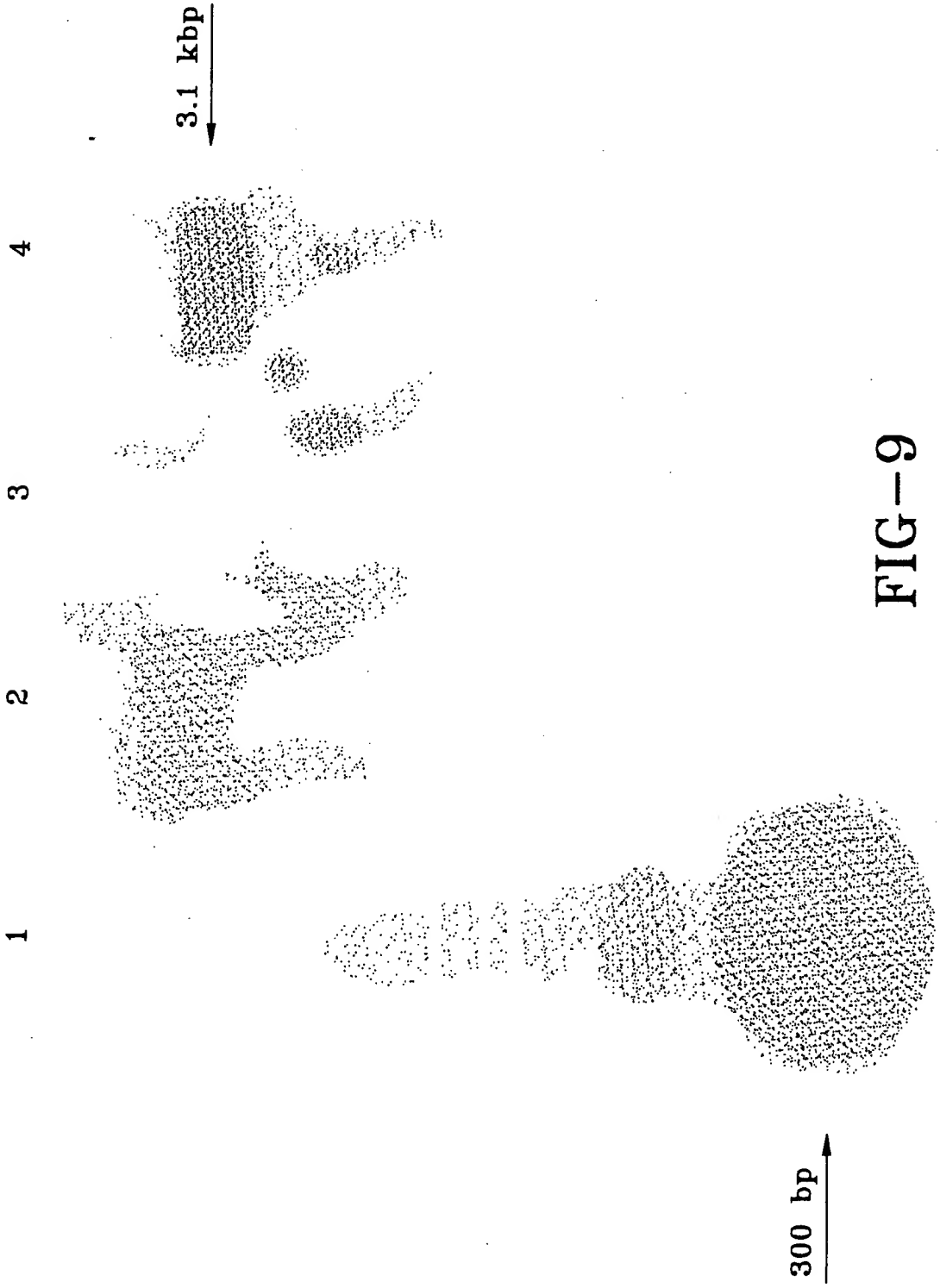


FIG-9

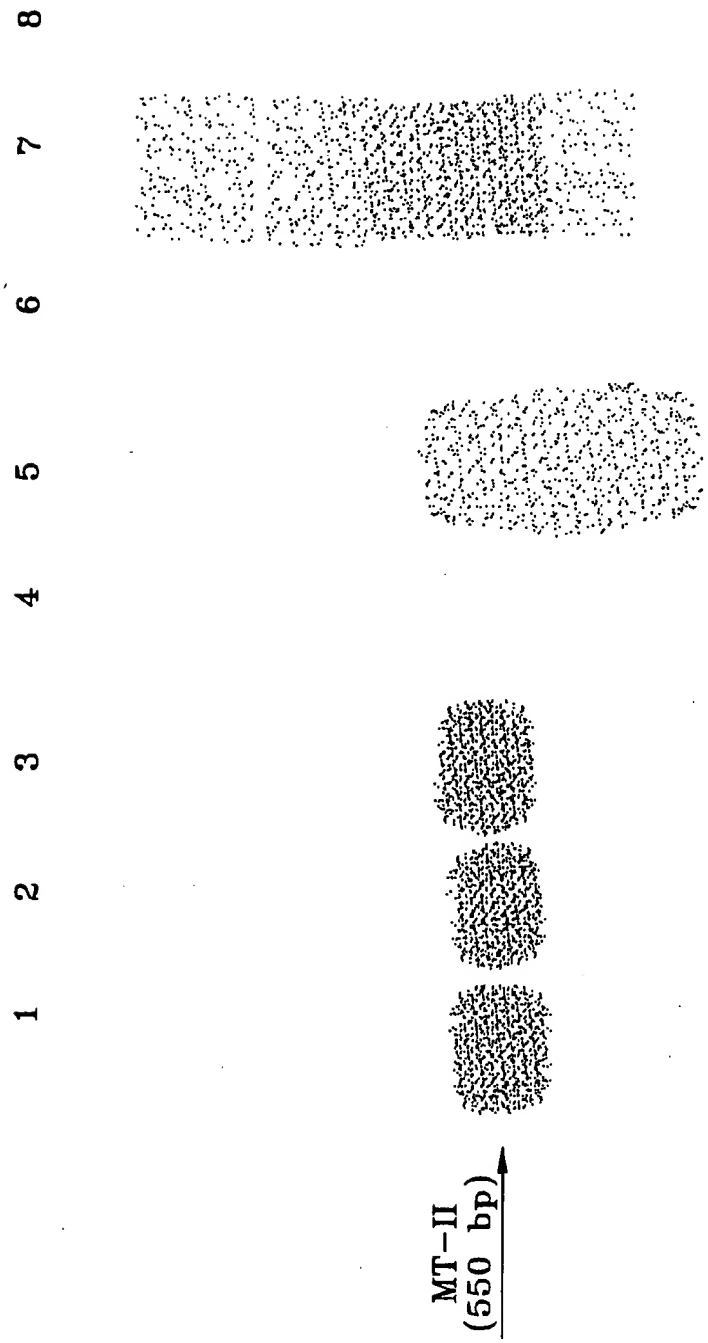


FIG-10

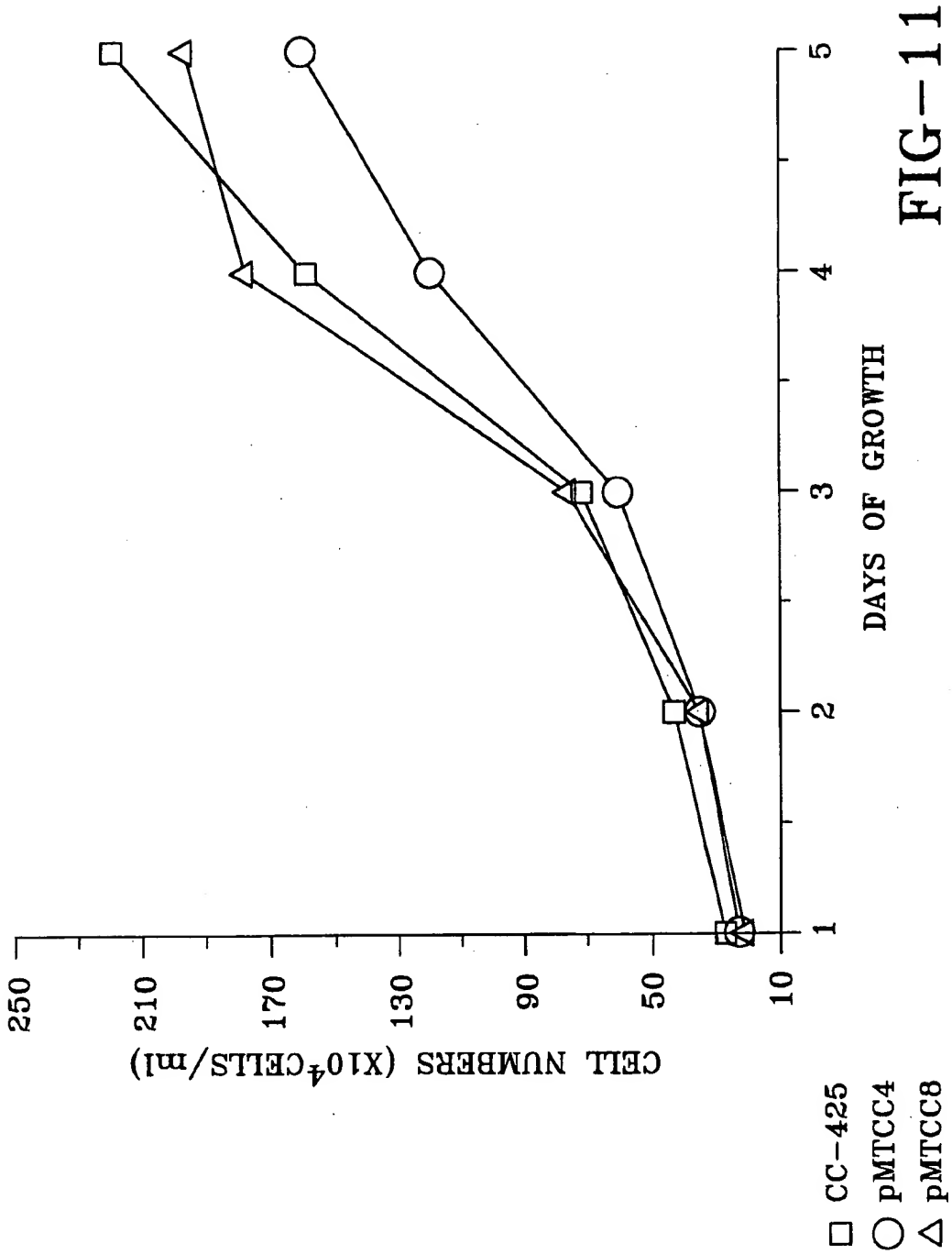

CELL NUMBERS (X10⁴CELLS/ml)
250
210
170
130
90
50
10
1
2
3
4
5
DAYS OF GROWTH
□ CC-425
○ pMTCC4
△ pMTCC8

FIG-11

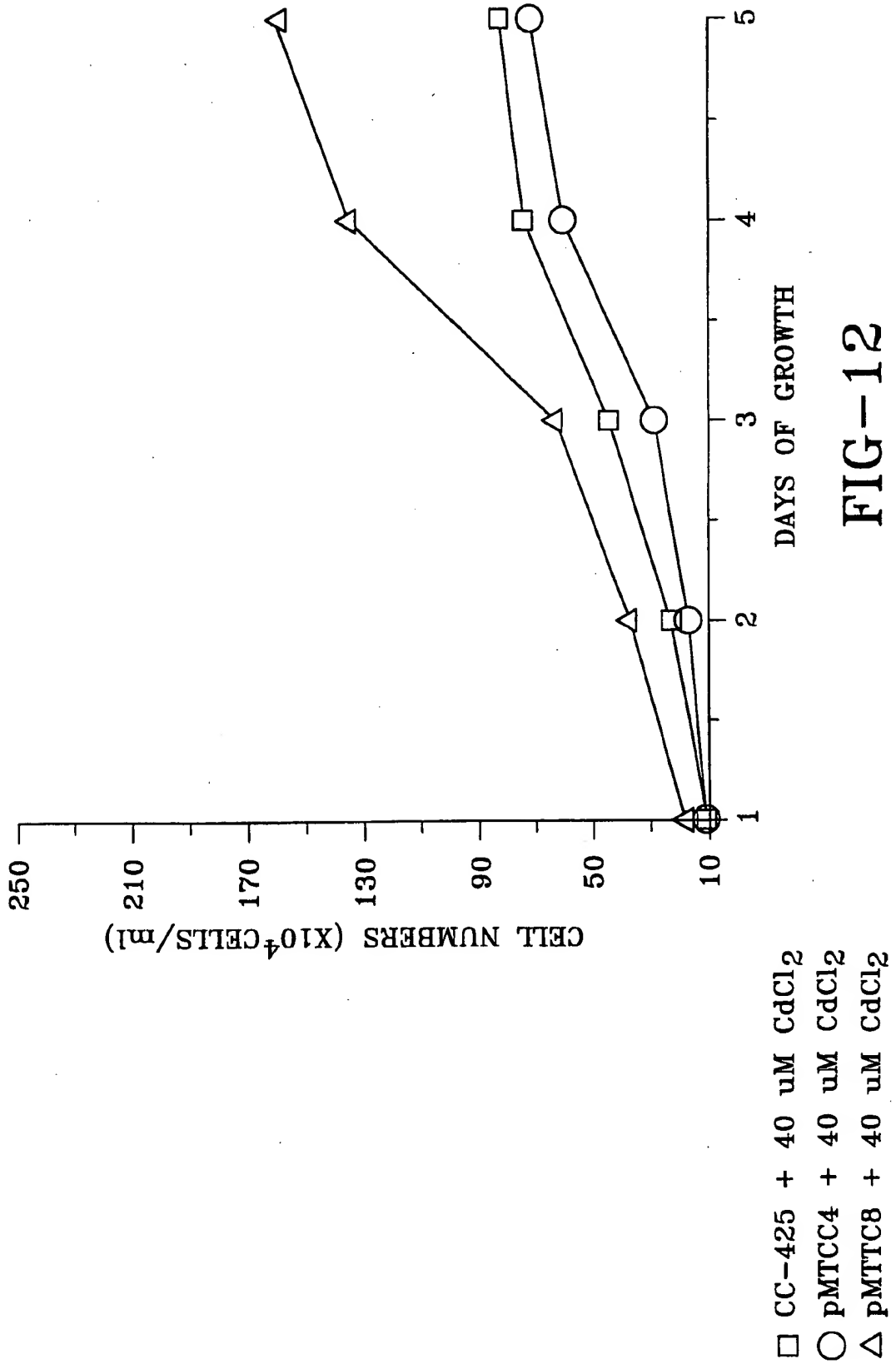
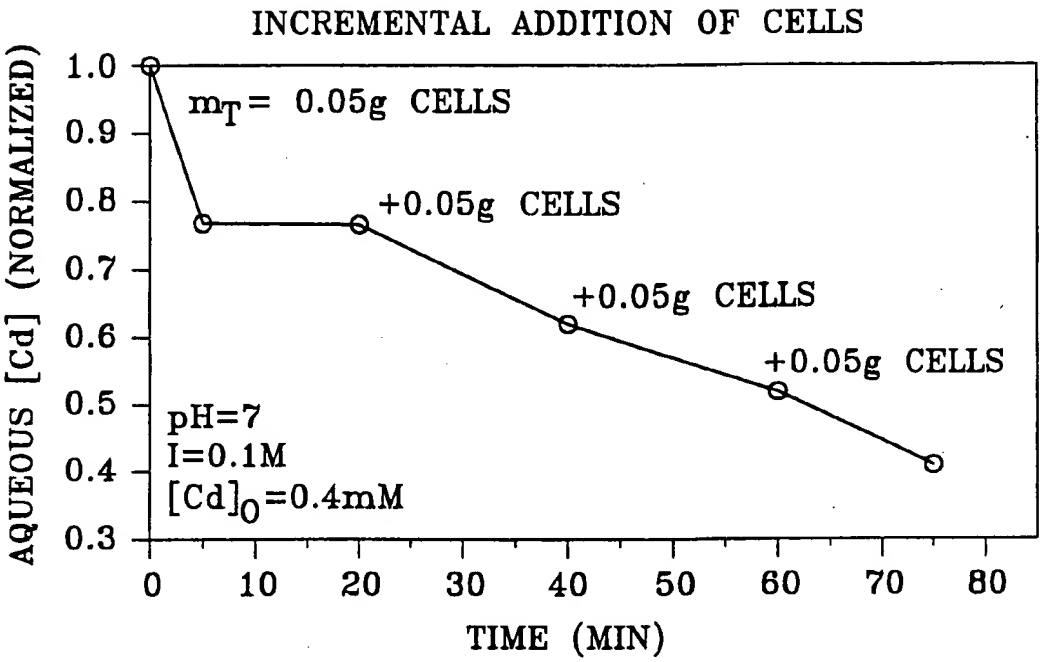
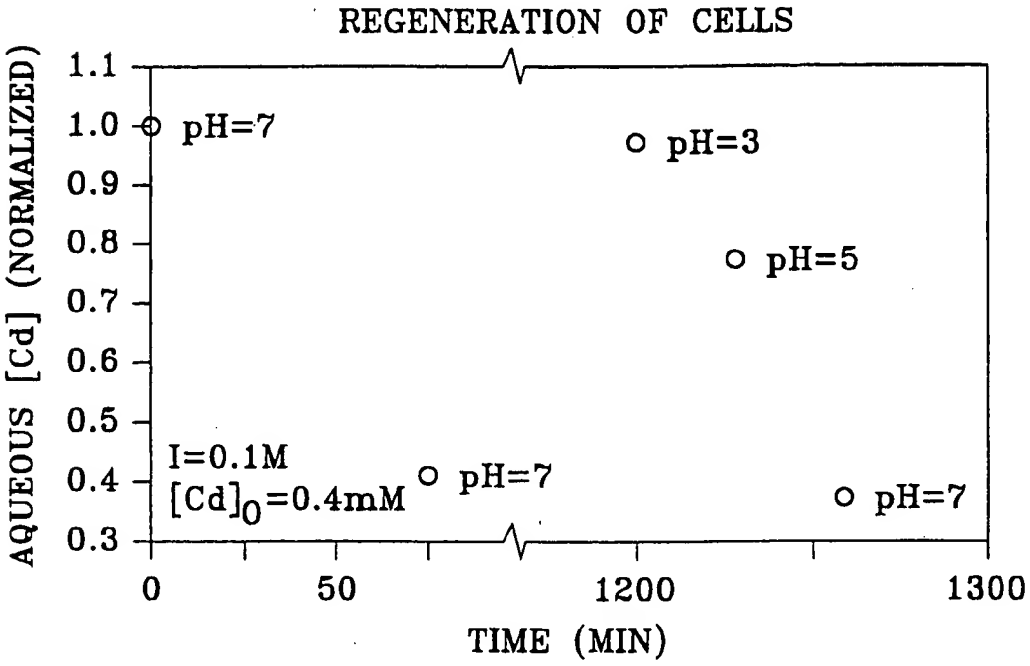

CELL NUMBERS (X10⁴CELLS/ml)
250
210
170
130
90
50
10
1
2
3
4
5
DAYS OF GROWTH
□ CC-425 + 40 uM CdCl2
○ pMTCC4 + 40 uM CdCl2
△ pMTTC8 + 40 uM CdCl2

FIG-12


INCREMENTAL ADDITION OF CELLS
AQUEOUS [Cd] (NORMALIZED)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
m_T = 0.05g CELLS
+0.05g CELLS
+0.05g CELLS
+0.05g CELLS
pH=7
I=0.1M
$[Cd]_0$=0.4mM
0
10
20
30
40
50
60
70
80
TIME (MIN)

FIG−13


REGENERATION OF CELLS
AQUEOUS [Cd] (NORMALIZED)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
pH=7
pH=3
pH=5
I=0.1M
$[Cd]_0$=0.4mM
pH=7
pH=7
0
50
1200
1300
TIME (MIN)

FIG−14

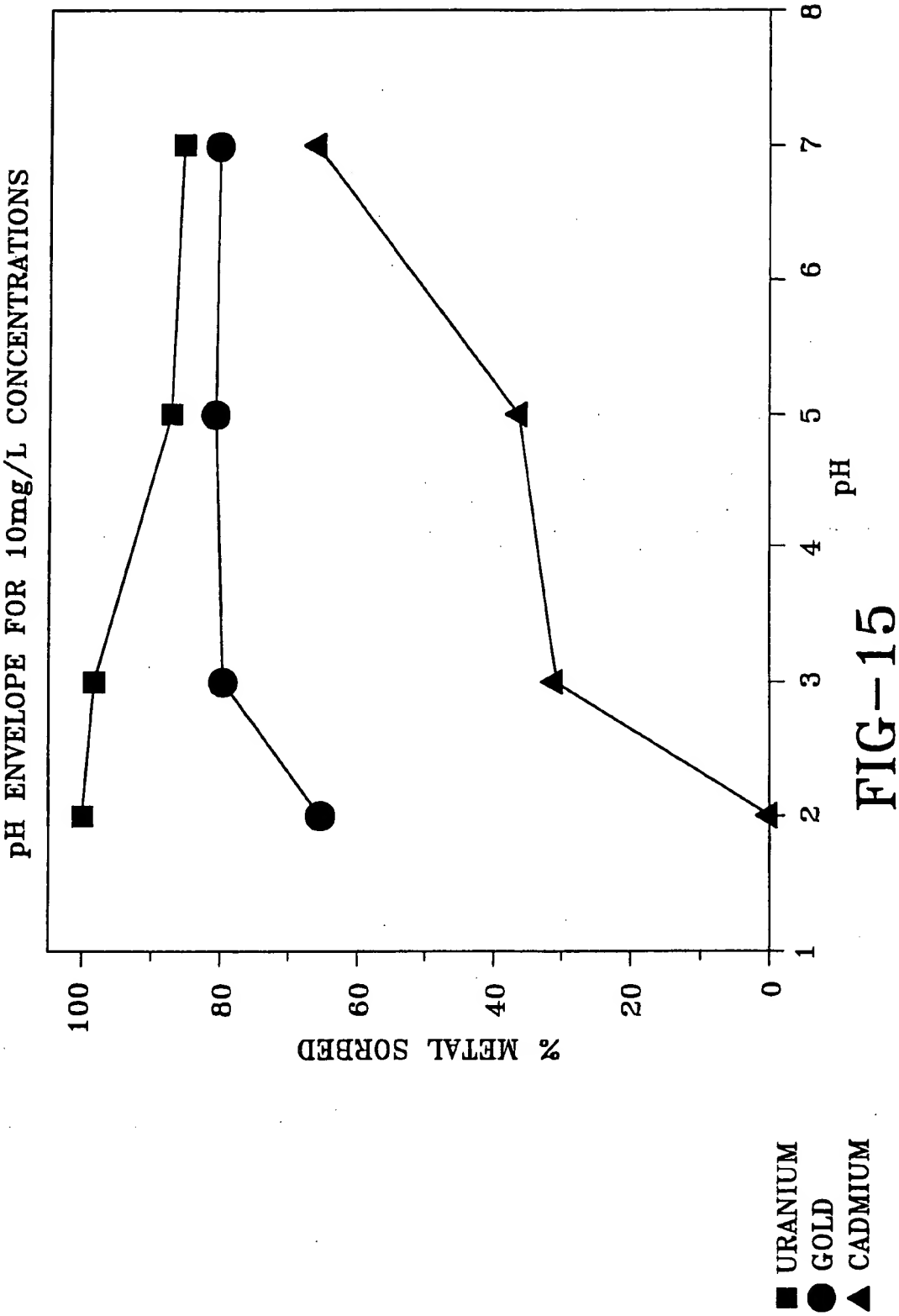

pH ENVELOPE FOR 10mg/L CONCENTRATIONS
% METAL SORBED
0
20
40
60
80
100
1
2
3
4
5
6
7
8
pH
URANIUM
GOLD
CADMIUM

FIG-15

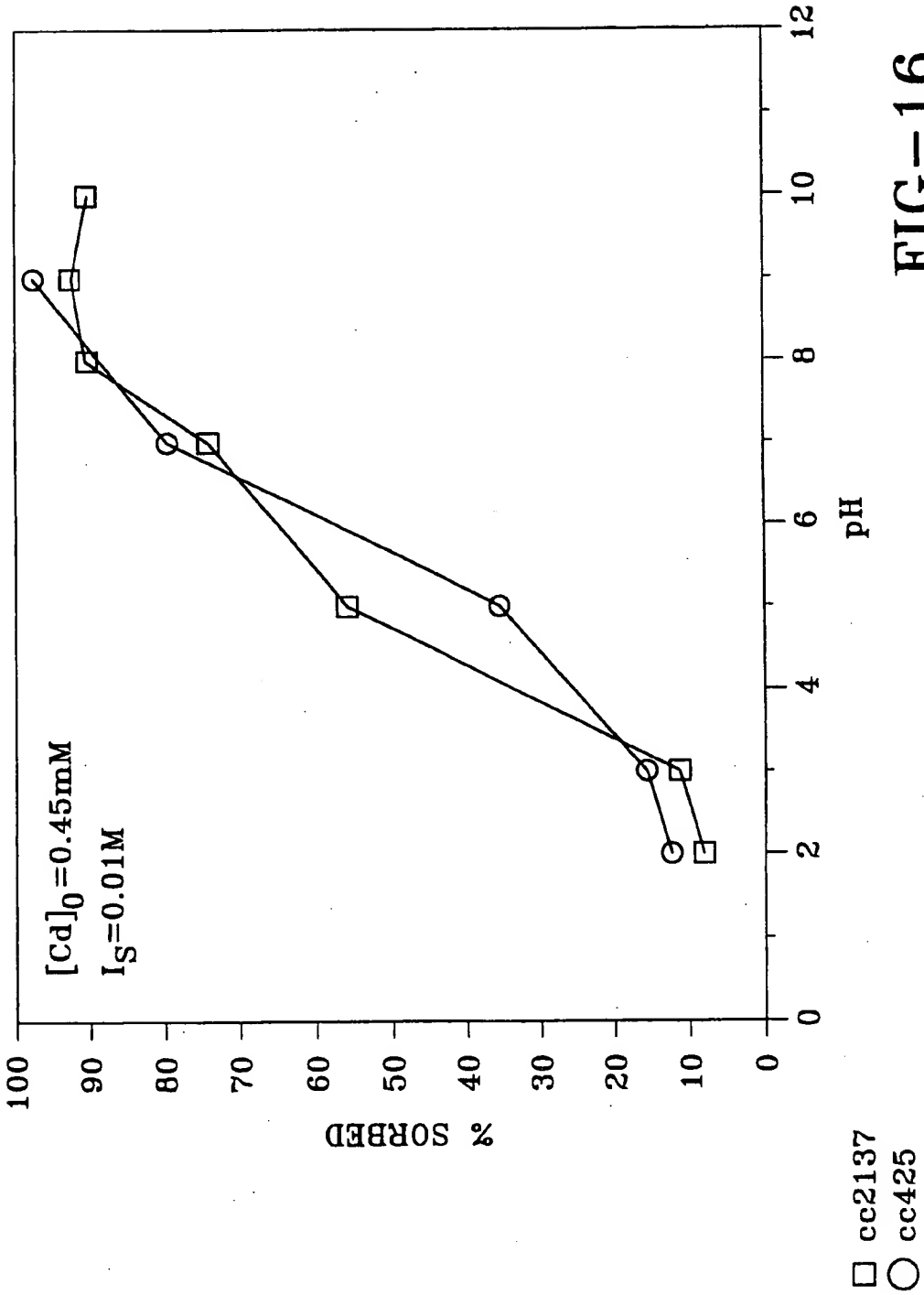


FIG-16

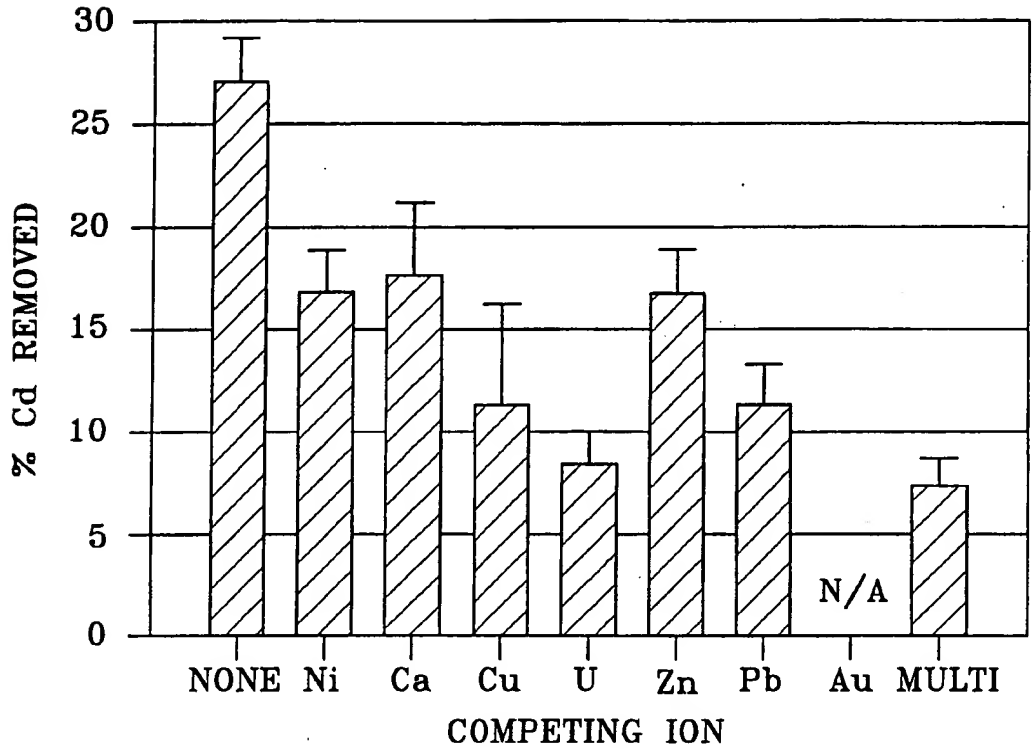

30
25
20
15
10
5
0
% Cd REMOVED
N/A
NONE Ni Ca Cu U Zn Pb Au MULTI
COMPETING ION

FIG-17

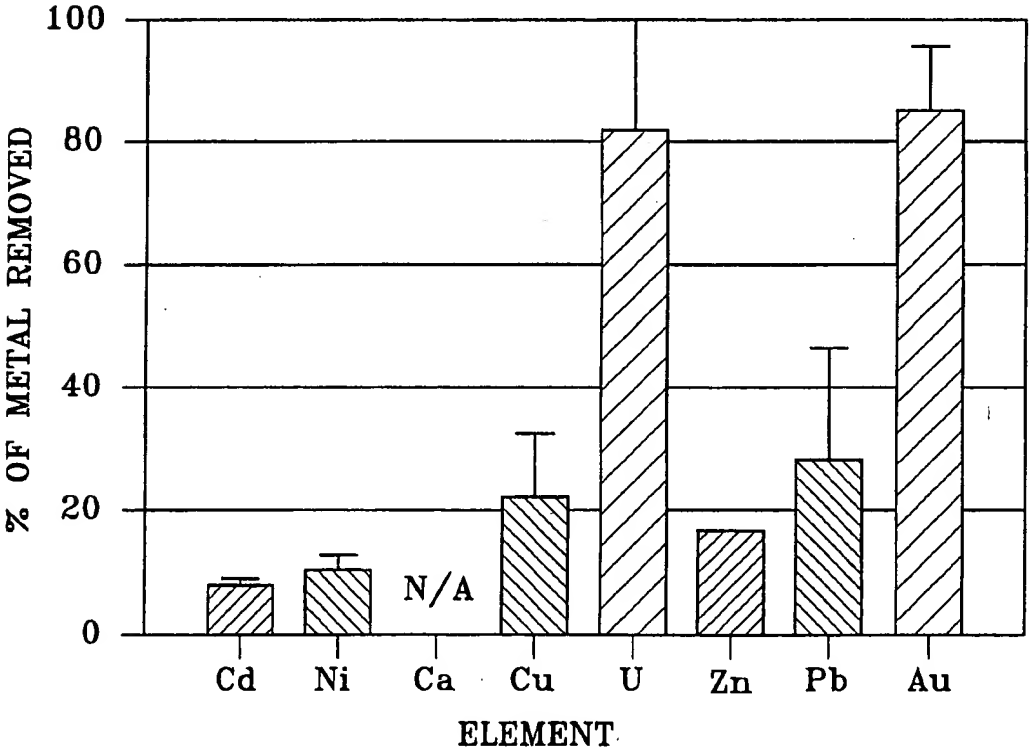

100
80
60
40
20
0
% OF METAL REMOVED
N/A
Cd Ni Ca Cu U Zn Pb Au
ELEMENT

FIG-18

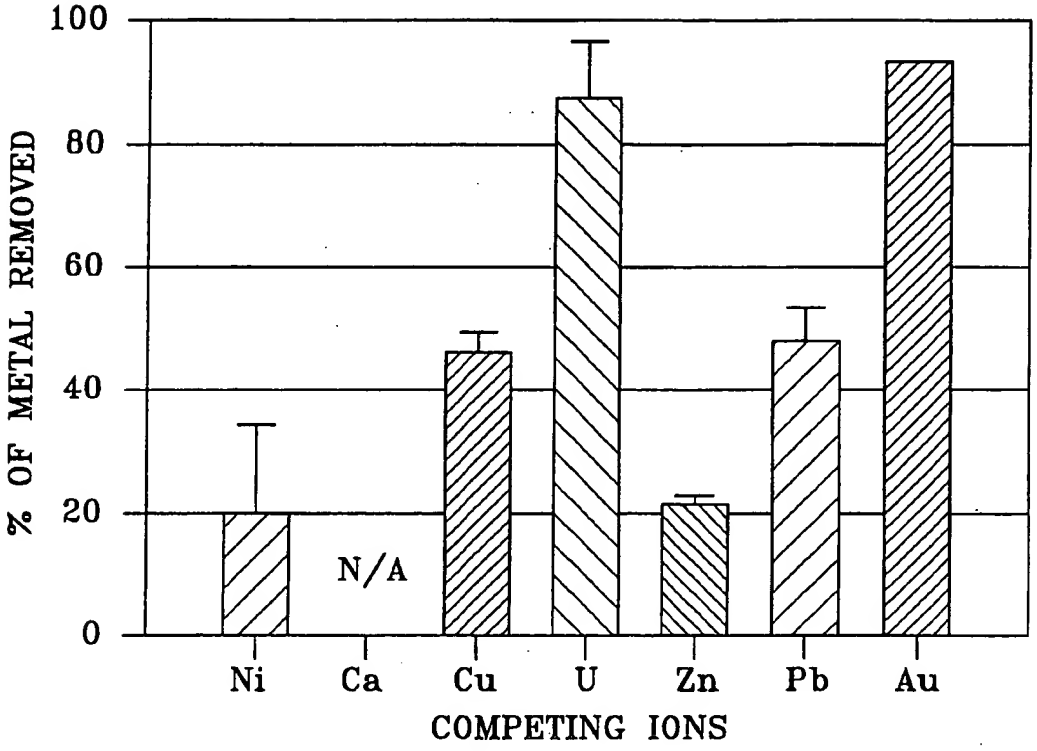

100
80
60
40
20
0
% OF METAL REMOVED
N/A
Ni
Ca
Cu
U
Zn
Pb
Au
COMPETING IONS

FIG-19

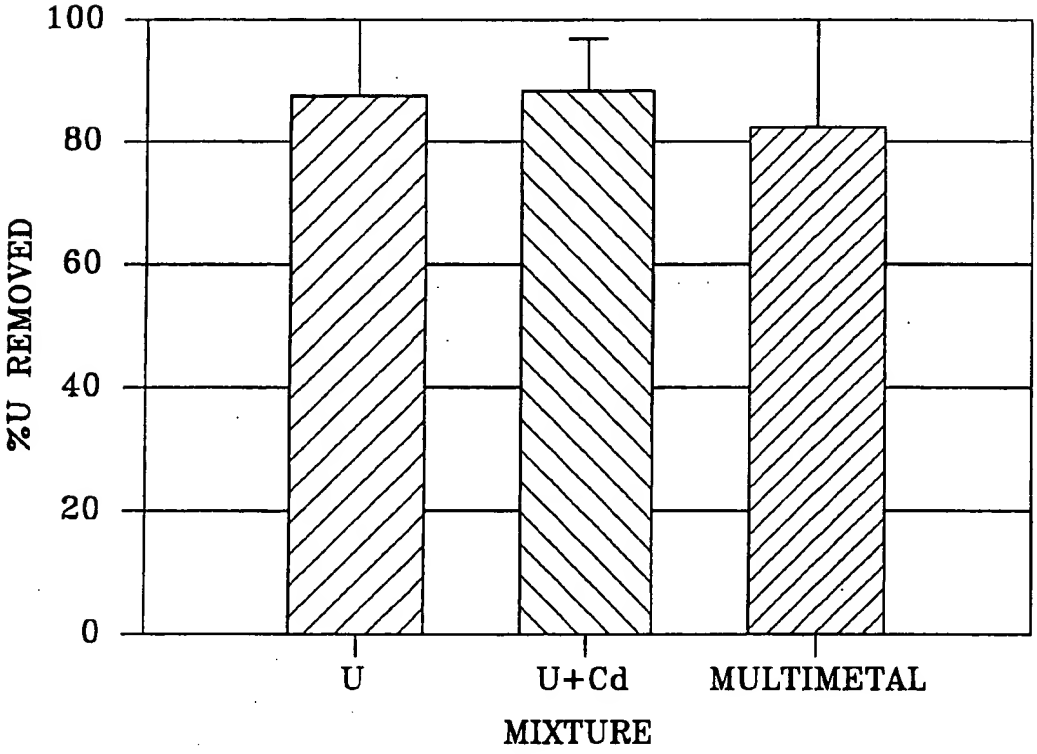

100
80
60
40
20
0
%U REMOVED
U
U+Cd
MULTIMETAL
MIXTURE

FIG-20

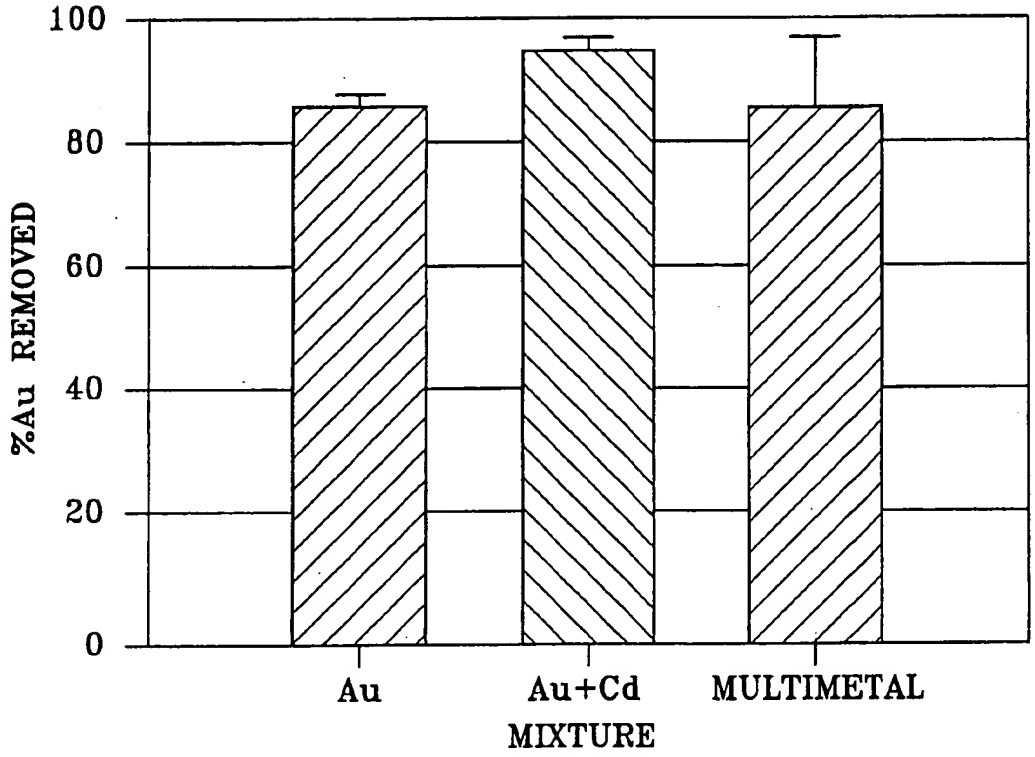

100
80
60
40
20
0
%Au REMOVED
Au
Au+Cd
MULTIMETAL
MIXTURE

FIG-21

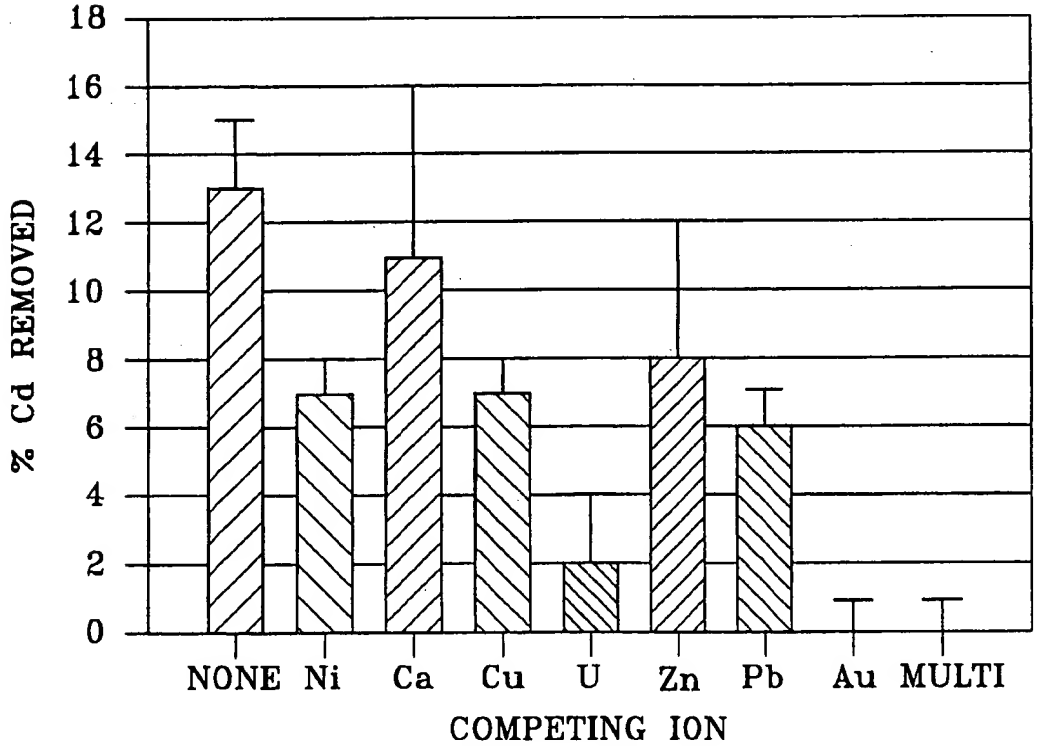

18
16
14
12
10
8
6
4
2
0
% Cd REMOVED
NONE
Ni
Ca
Cu
U
Zn
Pb
Au
MULTI
COMPETING ION

FIG-22

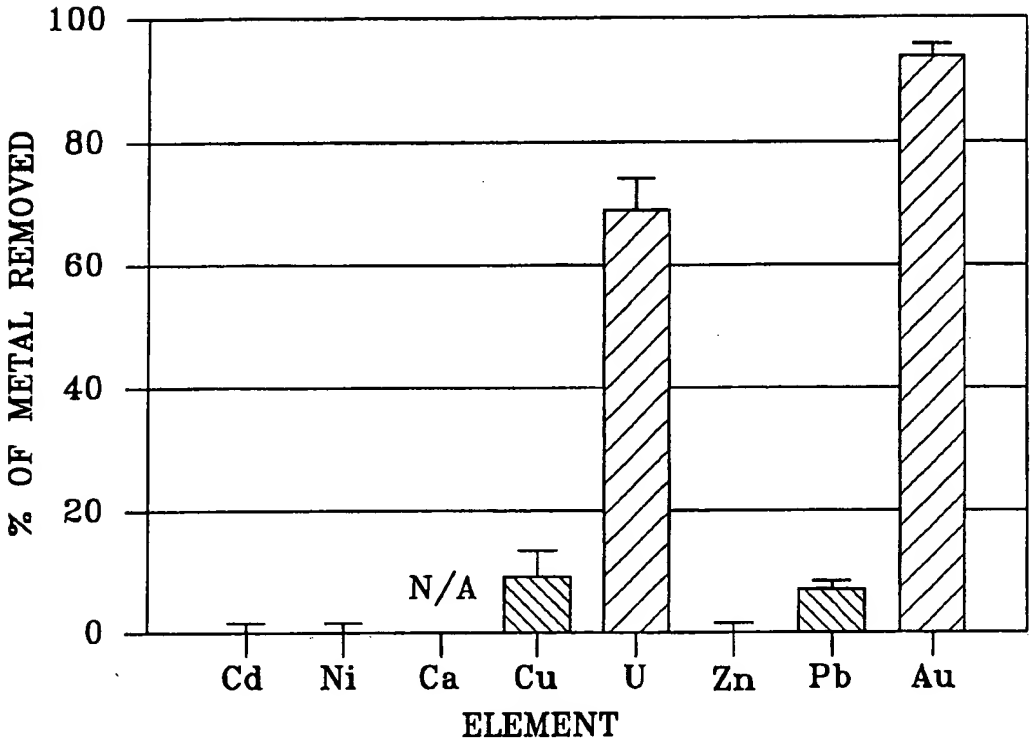

100
80
60
40
20
0
% OF METAL REMOVED
N/A
Cd
Ni
Ca
Cu
U
Zn
Pb
Au
ELEMENT

FIG-23

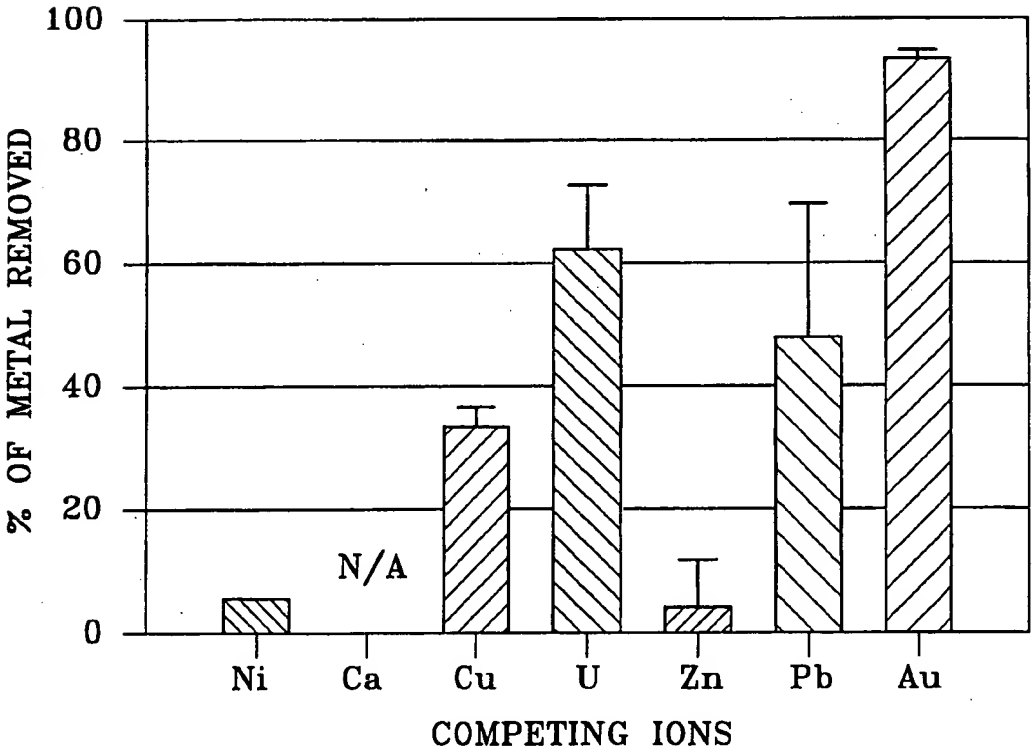

100
80
60
40
20
0
% OF METAL REMOVED
N/A
Ni
Ca
Cu
U
Zn
Pb
Au
COMPETING IONS

FIG-24

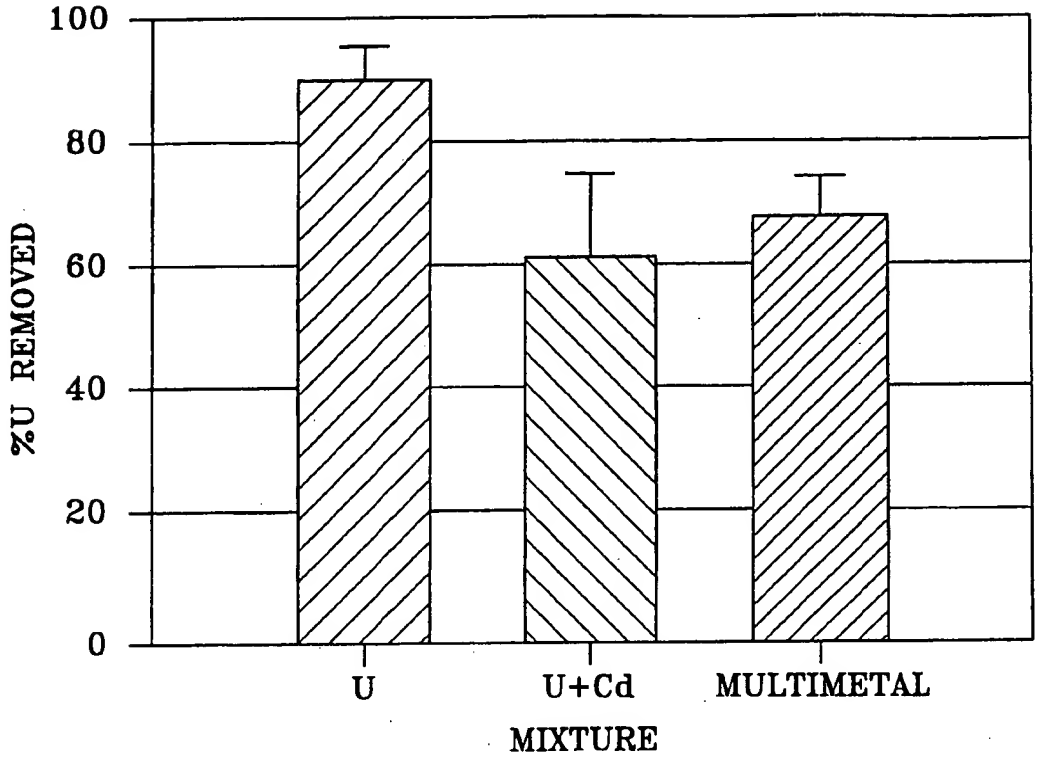

100
80
60
40
20
0
%U REMOVED
U
U+Cd
MULTIMETAL
MIXTURE

FIG-25

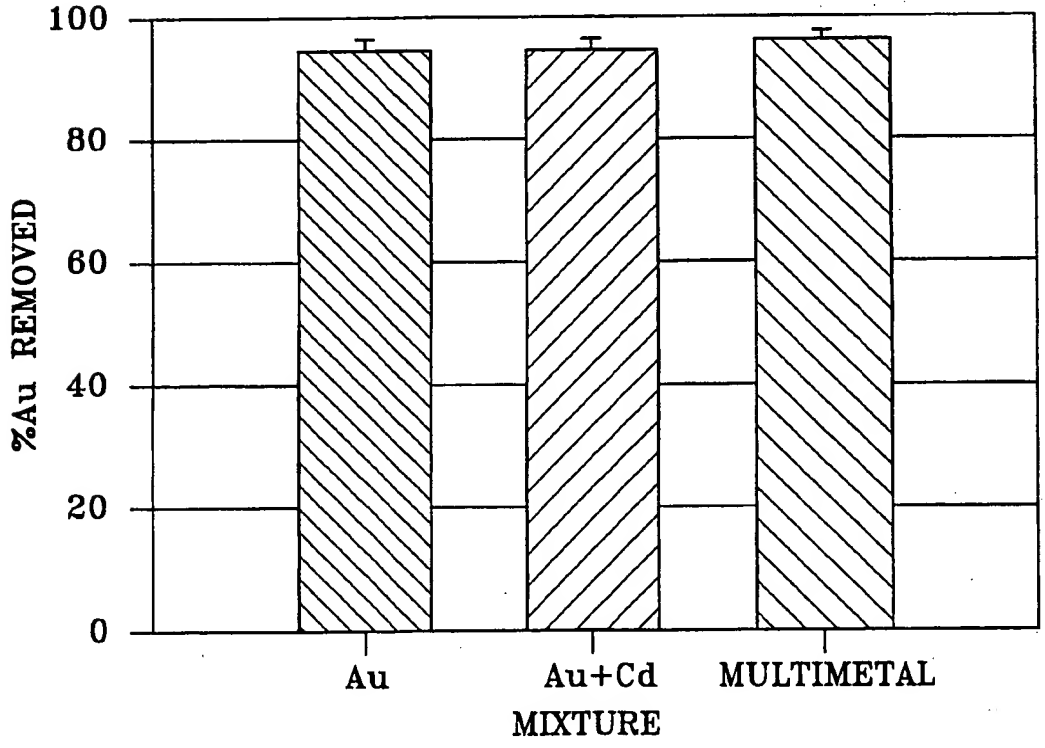

100
80
60
40
20
0
%Au REMOVED
Au
Au+Cd
MULTIMETAL
MIXTURE

FIG-26